Flavour Aspects of Pea and its Protein Preparations in Relation to Novel Protein Foods

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To my beloved parents

and grandma

2000-2004

ABSTRACT

Flavour involves aroma and taste, which are important characteristics for the acceptability of novel protein foods (NPFs). NPFs can be produced from pea protein isolates containing vicilin and legumin. Peas contain both non-volatile and volatile flavour compounds that influence taste and aroma. The non-volatile compounds consist of 2 saponins, whereas the volatile organic compounds (VOCs) in peas belong to 3 main groups, the aldehydes, ketones and alcohols. Peas contain DDMP saponin and saponin B, which are oleanane type saponins that are most common in nature. DDMP saponin is labile and can be converted to saponin B. DDMP saponin was stable at around pH 7 and at ethanol concentrations > 30% (v/v), but lost its stability at acidic and alkaline pHs, and at temperature > 30 ^{o}C. Both DDMP saponin and saponin B have a bitter taste, but the former is significantly more bitter than the latter. The contents of DDMP saponin and saponin B are variety dependent and differed among 16 pea varieties (0.7-1.5 g/kg and 0-0.4 g/kg, respectively). DDMP saponin predominates over saponin B in all varieties; in 2 varieties, DDMP saponin was the only saponin present. Pea flour contains the highest amount of VOCs compared to its protein preparations, and the type and amount of VOCs released are influenced by protein purification and pH. Pea vicilin showed affinity for exogenous aldehydes and ketones, whereas legumin showed affinity only for the aldehydes. Vicilin preparations contained non-protein components, lipids and carbohydrates, which exhibit greater affinity for VOC than vicilin itself. Overall, the results obtained in this thesis have provided essential knowledge on flavour aspects, especially with respect to off-flavours, in the development of NPFs.

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Chapter 1

General Introduction

Novel Protein Foods and PROFETAS

The idea to develop novel protein foods (NPFs) based on plant proteins, as an alternative to meat, arises with the aim to reduce environmental burdens brought about by existing meat production systems, which inefficiently use large amount of energy, land and raw materials. For NPFs to be successful, they have to be nutritionally (*i.e.* in terms of protein quality) similar to meat and should have a flavour and texture appealing to consumers. Existing NPFs produced from non-animal protein sources, such as soy, are still considered to have an inferior flavour and texture, and hence, unappealing to most consumers. Based on these principles, the interdisciplinary program, PROFETAS (PROtein Foods Environment Technology And Society) was initiated and established. PROFETAS aims to provide knowledge through the assessment of technological feasibility, environmental sustainability and societal acceptability of producing NPFs based on pea proteins. Peas were the protein source of choice within the PROTETAS framework because of their high protein content and their suitability for cultivation in Europe.

Peas (*Pisum sativum* L.) and NPFs

Peas belong to the family of *Leguminosea*, which also include a number of edible beans such as soybeans and groundnuts, which are used as staple food. Over the years, peas have become increasingly important in Europe as a source of protein for both animal feed as well as for human food (Guillaume, 1977). Pea based ingredients have been used in vegetarian products such as burgers and sausages (Anonymous, 1998), and in pea snacks such as fried peas coated with wheat and/or rice flour mixed with sugar and other seasonings, or extruded fried green pea flour products flavoured with seasonings (Jambunathan *et al*., 1994).

The steadily growing vegetarian market has been facing the constant challenge of substituting meat with proteinaceous food products that not only have eating characteristics, such as texture and flavour, similar to those of meat, but also its nutritional adequacy (Lightowler *et al*., 1998; Davies & Lightowler, 1998). In recent years, ingredients from leguminous protein sources with brand names, such as Arrum (made from yellow peas and wheat gluten), have been used to formulate new products, *e.g*. NPFs (Davies & Lightowler, 1998; Zhu *et al*., 2004).

Composition of pea flour and derived fractions

The protein content of pea seeds is influenced by both genetic and environmental factors (Reichert & MacKenzie, 1982; Owusu-Ansah & McCurdy, 1991). Peas have a protein content that ranges

from 20-25% (Casey, 2003), a high starch content from 33-50% (Gatel & Grosjean, 1990), but are low in fat. Pea protein is a good source of essential amino acids, having a high lysine content, but is limiting in tryptophan and in the sulphur-containing amino acids, methionine and cysteine (Leterme *et al*., 1990). Pea flour is the main ingredient from peas and is obtained by milling pea seeds. This pea flour may be further processed into pea concentrate by *e.g*. air-classification, a dry processing method that removes the lighter starch granules from the heavier protein particles (Owusu-Ansah & McCurdy, 1991). Pea isolate, on the other hand, is obtained through aqueous extraction of pea flour followed by isoelectric precipitation of the extract. The estimated compositions of some pea (protein) preparations used as food ingredients are shown in **Table 1**.

Table 1: Composition of pea protein preparations*

Component	Pea flour (w/w)*	Pea concentrate (w/w)*	Pea isolate (w/w)*
Protein (N x 5.7)	25	47	80
Starch	56	7	3
Fat	1	4	2
Fibre	2	3	1
Ash	3	6	4

* Data taken from Sosulski and McCurdy (1987)

Pea proteins

Of the 25% of protein in pea flour, 70-80% consists of salt-extractable globulins and water-soluble albumins (Derbyshire *et al*., 1976). The globulins, which mainly consist of legumin and vicilin, account for 65-80% of the extractable proteins. Pea legumin, the 11S protein, is a protein of ~60 kDa that is proteolytically cleaved into a basic 20 kDa and an acidic 40 kDa polypeptide, interlinked by a disulphide bridge. These subunits assemble into a hexamer at pH 7-9 (Guéguen *et al*., 1988). As there are a number of legumin precursors originating from several gene families, different legumin polypeptides have been identified, *e.g*. 4-5 acidic and 5-6 basic polypeptides. The sizes of these polypeptides range from 38-40kDa for the acidic polypeptides, and from 19-22 kDa for the basic polypeptides (Krishna *et al*., 1979; Casey, 1979; 2003).

Pea vicilin, the 7S protein, is produced as a precursor of ~50 kDa which assembles into a trimer *in vivo* (Gatehouse *et al*., 1981). As specified by the coding sequence of the vicilin genes, vicilin can be cleaved at one or two sites, *i.e*. the α-β and the β-γ processing site (Gatehouse *et al*., 1983).

Cleavage at the α-β site produces fragments of 19 and 30 kDa, whereas that at the β-γ site produces fragments of 12.5 or 16 and 33 kDa. Cleavage at both sites produces fragments of 12.5, 13.5 and 16 or 19 kDa. These small fragments are only apparent under dissociating conditions, such as in the presence of sodium dodecyl sulphate (Gatehouse *et al*., 1981), and lead to the extensive heterogeneity observed for vicilin.

The ratio of vicilin to legumin varies from one pea genotype to another (Casey, 2003) and may range from 0.5 to 1.7, with a mean of 1.1 (Schroeder, 1982). Pea legumin and vicilin exhibit similarities in amino acid composition, molecular mass as well as subunit structure, to the soy proteins, glycinin and β-conglycinin (Derbyshire *et al*., 1976). A comparison of the rheological properties of pea legumin and soy glycinin has shown that both proteins have the same physical and chemical driving forces during gelation (O'Kane, 2004). The ability of proteins to form gels is important for the texture of food products and gelling properties have been investigated in another PROFETAS study by O'Kane (2004).

Flavour properties and perception

In addition to texture, flavour is another important parameter for the success of NPFs development. Flavour is a combination of taste, aroma, texture and trigeminal responses. Taste involves the sensation of sweet, sour, bitter, salty and umami, and is associated with the receptors on the tongue. Aroma sensation arises from a small area in the nasal cavity and covers a broad spectrum of approximately 10,000 different odours, which are the volatile components (Laing & Jinks, 1996; Reineccius, 1994). Texture or mouthfeel is the perception of the structure of food, such as smoothness, coarseness and regularity. The trigeminal responses result from the cranial nerves (which relate to the brain) in response to tactile (sense of touch) or temperature stimuli, such as the heat of spices or the cooling effect of menthol. Compounds responsible for taste are generally non-volatile at room temperature and they only interact with the taste receptors on the tongue. Aroma substances, on the other hand, are volatile compounds perceived by the odour receptor sites of the smell organ. They reach the receptor sites when drawn in through the nose (orthonasal) or via the throat after *e.g*. being released by chewing (retronasal) (Belitz & Grosch, 1999). Both volatiles (*e.g*. aldehydes and ketones) and non-volatiles (*e.g*. saponins) are major components contributing to the flavour of pea ingredients and therefore, to NPFs, and are the subject of study in this thesis.

Flavour components of pea

(A) Volatile organic compounds (VOCs) in green peas

The VOCs belonging to the class of aldehydes, ketones and alcohols, in common beans (*e.g*. lima beans and mung beans), lentils and split peas (Lovegren *et al*., 1979) were found to be similar to those VOCs found in Southern peas (*e.g*. black-eyed peas and cream peas) (Fischer *et al*., 1979). Green peas are known to have a 'green taste', which is due to a combination of several classes of VOCs such as aldehydes, ketones and alcohols within a matrix of protein and carbohydrate. These VOCs are, to a large extent, generated by oxidation of unsaturated fatty acids (Sessa & Rackis, 1976) and can also be synthesised via enzymatic reactions (Makower & Ward, 1950) by pea enzymes, such as catalases and peroxidases (Lee & Wagenknecht, 1958). The VOCs are either odour active themselves or can interact with other food components to release odour active VOCs.

Peas after harvesting, processing (*e.g*. de-hulling) or storage usually contain alcohols (methanol, ethanol & hexanol) as the dominating volatile components (Murray *et al*., 1976). Aldehydes and ketones, which are present in smaller amounts, are usually of greater significance because they possess stronger aromas (Murray *et al*., 1976). Hexanal, for example, is a significant odorous compound present in considerable amounts in frozen peas, and it is responsible for the "hay-like" off-flavour in these peas (Murray *et al*., 1976). Sulphur containing compounds (Ralls *et al*., 1965), and aliphatic and aromatic hydrocarbons (Murray *et al*., 1976) also contribute to off-flavour of peas.

Three different 3-alkyl-2-methoxypyrazines have also been identified as natural aroma constituents in green peas, namely, 3-isopropyl-2-methoxypyrazine, 3-sec-butyl-2-methoxypyrazine and 3-isobutyl-2-methoxypyrazine (Murray & Whitfield, 1975; Murray *et al*., 1970, 1976; Jakobsen *et al*., 1998). These compounds are present in extremely low concentrations, but are the main compounds that contribute to the perceived 'green pea' aroma in peas (Murray & Whitfield, 1975; Murray *et al*., 1970, 1976). Although the type and quantity of VOCs present in peas are known (Makower & Ward, 1950; Lee & Wagenknecht, 1958; Ralls *et al*., 1965; Murray & Whitfield, 1975; Murray *et al*., 1970, 1976; Sessa & Rackis, 1976; Jakobsen *et al*., 1998), such knowledge, however, is not available for pea protein preparations, such as pea protein isolates. As pea protein isolate is the pea ingredient mostly used in food applications, it is desirable to have an inventory of its volatile composition.

(B) Non-volatiles

(i) Saponin structure and properties

Saponins are surface-active triterpene glycosides that occur in a wide variety of plants such as peanuts, lentils, lupins, alfalfa, oats and spinach (Oakenfull, 1981; Fenwick & Oakenfull, 1983; Price *at al*., 1987; Ayet *et al*., 1996; Lasztity *et al*., 1998; Woldemichael *et al*., 2003; Oleszek *et al*., 1990a/b; Huhman & Sumner, 2002). They are non-volatile compounds that are known to have either a bitter (Price & Fenwick, 1984; Curl *et al*., 1985; Price *et al*., 1985; Reichert *et al*., 1986; Bishnoi & Khetarpaul, 1994) or a sweet taste (Grenby, 1991; Mizutani *et al*., 1994; Kennelly *et al*., 1995). Soybeans (*Glycine max*) and peas (*Pisum*) are the most significant sources of saponins in the human diet (Oakefull, 1981).

There are 2 types of saponins that have been isolated from peas, namely saponin B and DDMP saponin (Daveby *et al*., 1998) (**Figure 1**). Both saponins are *monodesmoside* saponins, having a sugar chain linked to only the C-3 position of their aglycones (soyasapogenol B) (Shiraiwa *et al*., 1991a/b; Yoshiki *et al*., 1998). DDMP saponin differs from saponin B in that it contains a DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) moiety at the C-22 position, which upon heating, is released as maltol (Kudou *et al*., 1993).

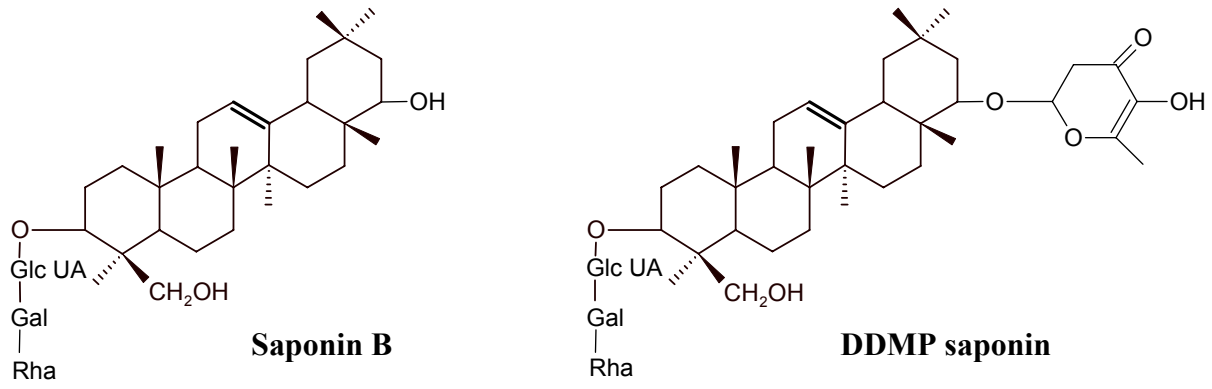


Figure 1: Structures of saponin B and DDMP saponins.

Legend*: 'Glc UA', 'Gal' and 'Rha' represent glucuronic acid, galactose and rhamnose, respectively.*

Being amphiphilic in nature, saponins can form stable foams and act as emulsifying agents, and thus have found application in beverages and confectionery, as well as in cosmetics (Price *et al*.,

1987). Studies have shown that dietary saponins may reduce plasma cholesterol in animals (Oakenfull *et al*., 1979; Topping *et al*., 1980) and bind bile acids (Topping *et al*., 1980). Other biological activities of saponins include antifungal (Oleszek *et al*., 1990b), anti-cancer (Konoshima *et al*., 1992), anti-AIDS (Sakurai *et al*., 2004) and oxygen scavenging activities (Yoshiki & Okubo, 1995).

Saponin B has been reported as the main saponin component in peas (Price & Fenwick, 1984; Curl *et al*., 1985; Price *et al*., 1985; 1988; Daveby *et al*., 1997; Kinjo *et al*., 1998), with a higher concentration in the hulls (Reichert *et al*., 1986; Bishnoi & Khetarpaul, 1994) and the protein fractions than in the pea flour (Curl *et al*., 1985). Processing steps such as sprouting, de-hulling, soaking and cooking, have shown to decrease the saponin contents in peas by 3-84% (Bishnoi & Khetarpaul, 1994). Recently, in addition to saponin B, DDMP saponin was identified in peas (Daveby *et al*., 1998), which was obtained using a mild extraction procedure. DDMP saponin has been found to be easily converted to saponin B under the influence of temperature and pH (Massiot *et al*., 1996; Okubo *et al*., 1996). However, the exact influence of these parameters on DDMP saponin stability is largely unknown and should thus be established, in order to be able to study the pea saponins in their native form.

(ii) Sensory properties of saponins

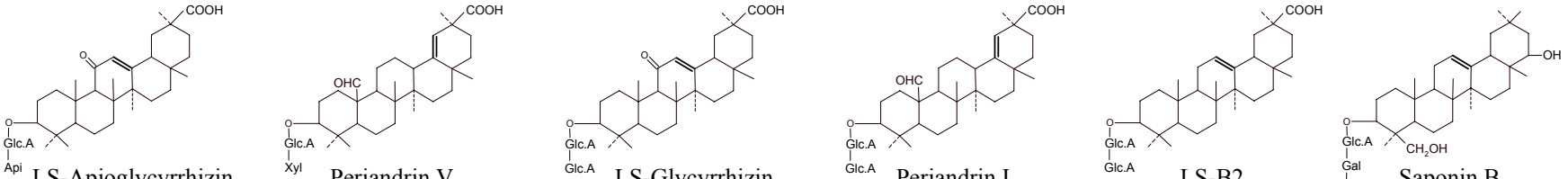
Saponins in pea flour and protein-rich pea fractions have been reported to be perceived as having a bitter and metallic taste (Price & Fenwick, 1984; Curl *et al*., 1985; Price *et al*., 1985; Reichert *et al*., 1986; Bishnoi & Khetarpaul, 1994). The intensity of this bitterness was also shown to be related to the saponin contents of these pea products (Price *et al*., 1985); pea flour had a higher bitterness rating than pea flour that contained half the amount of saponin. The undesirable bitter taste of saponins poses a major problem in the use of saponin containing plants and their products, such as peas, in food production (Davies & Lightowler, 1998). Although the sensory properties of saponin B are known, those of DDMP saponin are unknown. In addition, the intensity of bitterness of saponin B in comparison to common bitter compounds, *e.g*. caffeine, is also not known. Therefore, detailed knowledge about the sensory characteristics of the different pea saponins would provide essential information when using pea ingredients in food.

Many saponins that have been identified up to date were isolated because of their special characteristics such as bitterness or extreme sweetness. The structures of saponins from various

plants and their sensory characteristics are presented in **Figure 2** (Kasai *et al*., 1987; 1988; 1989; Kitagawa, 2002; Kinghorn & Soejarto, 2002). Liquorice saponins, such as glycyrrhizin and apioglycyrrhizin, have intense sweetness of 150-300 times sweeter than sucrose (Mizutani *et al*., 1994; Kitagawa, 2002). The periandrins I and V from the Brazilian liquorice (Hashimoto *et al*., 1980; 1982; 1983; Suttisri *et al*., 1993) are about 90-200 times sweeter than sucrose (Kinghorn & Soejarto, 2002). Both glycyrrhizins and periandrins have similar skeletal structure as saponin B, which is bitter in taste (Price & Fenwick, 1984), but differ in the type of substituents and/or sugar units attached to the skeleton. Some cucurbitane saponins are 77-250 times sweeter than sucrose, whereas others are tasteless or bitter, depending on the type of substituents and/or number of sugar residues attached to the skeleton (Takasaki *et al*., 2003; Kasai *et al*., 1987; 1988). Other known sweet saponins include the secodammarane saponins and the cycloartane saponins (Choi *et al*., 1989; Kennelly *et al*., 1996), which are 30-150 times sweeter than sucrose (Kennelly *et al*., 1995; Kinghorn & Soejarto, 2002).

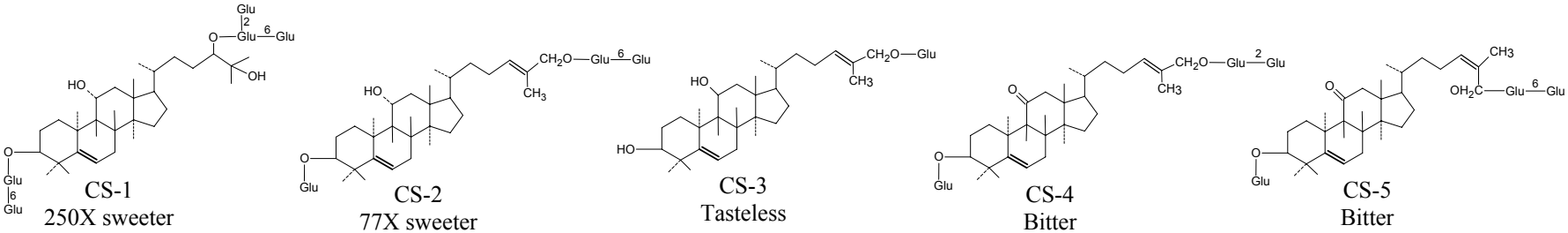
Apioglycyrrhizin differs from LS-glycyrrhizin only in one sugar residue, apiose, and is twice as sweet as LS-glycyrrhizin. Similarly, periandrin V differs from periandrin I by one sugar residue, xylose. The saponins within each group of the secodammarane and the cycloartane also differ in sweetness due to different sugar residues. The presence of these sugar residues may be an explanation for the sweetness of these saponins. However, the carbonyl group at C11 seems to be of more importance, as its absence leads to the conversion of sweetness to bitterness. Of the many known liquorice saponins, LS-B2 was found to be bitter (Kitagawa, 2002). This bitter liquorice saponin, named 11-deoxoglycyrrhizin, differs from the sweet LS-glycyrrhizin in that it does not have a carbonyl at C11. The substituent-taste relationships observed for the sweet and bitter cucurbitane saponins seem in contrast to that observed for the liquorice saponins. The carbonyl at C11 of cucurbitane saponins is responsible for their bitterness (CS-4 and 5) and the 11-deoxo-cucurbitane saponins (CS-1 and 2) are sweet (Kasai *et al*., 1987; 1988). These results may indicate that the oxygen function at C11 of each skeleton plays a role in taste, be it bitter or sweet, and it also depends on the rest of the substituents and/or the number of sugar residues in the molecule. In addition to substituents, the stereochemistry of saponin structures also affects taste properties (Kitagawa, 2002). The type of substituents and sugar residues located at the different carbon positions of a skeleton contribute to the taste of a saponin, but it is difficult to know if a saponin is sweet or bitter simply based on their structural characteristics.

Liqourice saponins



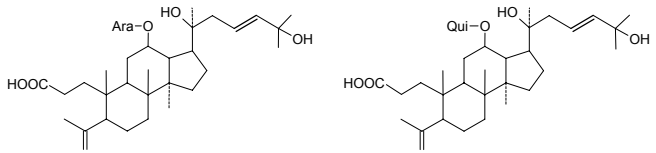
LS-Apioglycyrrhizin 300X Sweeter | Periandrin V 200X Sweeter | LS-Glycyrrhizin 150X Sweeter | Periandrin I 90X Sweeter | LS-B2 Bitter | Saponin B Bitter

Cucurbitane saponins



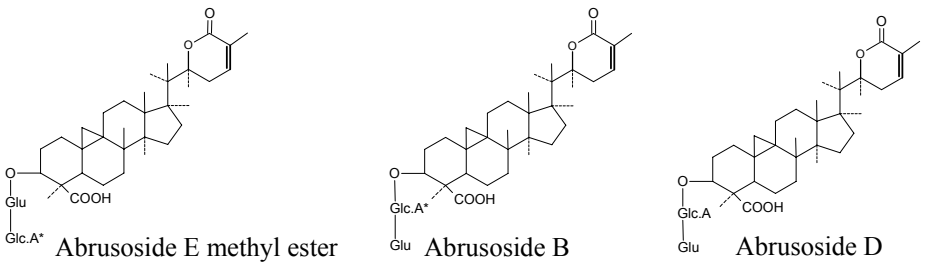
CS-1 250X sweeter | CS-2 77X sweeter | CS-3 Tasteless | CS-4 Bitter | CS-5 Bitter

Secodammarane saponins



Pterocaryoside B 250X sweeter | Pterocaryoside A 50X sweeter

Cycloartane saponins



Abrusoside E methyl ester 150X sweeter | Abrusoside B 100X sweeter | Abrusoside D 75X sweeter

Figure 2: Structures of four classes of sweet and bitter saponins. LS: Liquorice saponin; CS: Cucurbitane saponin; Api: Apiose; Ara: Arabinose; Gal: Galactose; Glc.A: Glucoronic acid; Glc.A*: Glucoronic acid methyl ester; Glu: Glucose; Qui: Quinovose; Rha: Rhamnose; Xyl: Xylose. Number(s) in sugar chains indicate the linkage between 2 sugar residues. Sweetness is in comparison with sucrose.

(iii) Saponins identification and quantification

Various methods of identification and quantification of saponins in peas have been employed, which include thin layer chromatography (TLC) (Fenwick & Oakenfull, 1983; Curl *et al*., 1985; Price *et al*., 1985; 1986; Khalil & El-Adawy, 1994; Ohana *et al*., 1998), reversed-phase high performance liquid chromatography (RP-HPLC) with UV detection (Price *et al*., 1986; 1988; Tsurumi *et al*., 1992; Daveby *et al*., 1997; 1998; Kinjo *et al*., 1998), colorimetric methods (Bishnoi & Khetarpaul, 1994) and haemolysis (Khalil & El-Adawy, 1994). The results obtained using these different methods give rise to huge deviations in estimated pea saponin contents. These differences may have arisen from either the extraction procedures or the applied analytical methods. RP-HPLC with evaporative light scattering detection (ELSD) has been used to separate and detect saponins without the need for derivatisation (Ireland & Dziedzic, 1986; 1987) from ginseng (Park *et al*., 1996) and soybean (Berhow *et al*., 2000). RP-HPLC-ELSD has the advantage over the above-mentioned methods, as it is a sensitive method that allows the separation and quantification of all intact saponins in one single gradient elution, as response factors of compounds from the same class vary only slightly.

(iv) Factors influencing saponin contents

The saponin content in peas is known to be influenced by several factors, such as seed development (Fenwick & Oakenfull, 1983; Khohhar & Chauhan, 1986; Sharma & Sahgal, 1992; Ayet *et al*., 1997; Daveby *et al*., 1997) and variety (Fenwick & Oakenfull, 1983; Khohhar & Chauhan, 1986; Shiraiwa *et al*., 1991c; Ruiz *et al*., 1997; Daveby *et al*., 1997; Duhan *et al*., 2001). Germination of legume seeds has been observed to cause an increase in saponin content by 17-56% (Ayet *et al*., 1997), whereas others have reported a decrease (15-84%) (Bishnoi & Khetarpaul, 1994; Sharma & Sahgal, 1992; Duhan *et al*., 2001). Saponin contents in peas have also been found to decrease by 50-75% during seed maturation (Daveby *et al*., 1997). For a number of different pea varieties, saponin contents ranging from 0.8 to 2.5 g/kg have been reported (Bishnoi & Khetarpaul, 1994; Daveby *et al*., 1997).

Protein-flavour interactions and their effects on flavour perception

(i) Protein-VOC interactions

Proteins, such as soy proteins and milk proteins, are known to interact with VOCs (Arai *et al*., 1970; Gremli, 1974; Frazen & Kinsella, 1974; King & Solms, 1979; Damodaran & Kinsella, 1980, 1981a/b, 1983; O'Keefe *et al*., 1991a/b; Andriot *et al*., 1999, 2000; Rogacheva *et al*., 1999;

Guichard & Langourieux, 2000). This flavour-binding property of proteins is of importance in the formulation of NPFs and other foods, in which the proteins may act as flavour carriers. The interaction between proteins and VOCs in aqueous systems mainly involves hydrophobic binding (Damodaran & Kinsella, 1980, 1981a/b, 1983; O'Neill & Kinsella, 1987b; Andriot *et al*., 2000) and is dependent on the extent of hydration of the protein molecules in the food (Damodaran, 1996). Non-polar VOCs such as aldehydes and ketones may interact primarily with the hydrophobic patches on the protein surfaces. Aldehydes are also known to be involved in covalent interactions (Plug & Haring, 1993; 1994). As hydrophobic interactions are the predominant interactions between non-polar VOC molecules and hydrated protein, factors such as surface hydrophobicity of the protein, pH, temperature, ionic strength, salt concentration, ethanol concentration, etc, will influence the retention of VOC to a protein (Druaux *et al*., 1995; Damodaran, 1996; Fischer & Widder, 1997). Apart from the changes in the charge on proteins, changes in pH may also influence the conformation of protein molecules, and hence affect protein-VOC interactions (Dumont & Land, 1986; Druaux *et al*., 1995; Damodaran, 1996; Lubbers *et al*., 1998; Andriot *et al*., 1999; Rogacheva *et al*., 1999; Guichard & Langourieux, 2000). Changes in temperature will also influence VOC retention when the protein molecules undergo significant temperature induced structural changes. Protein-VOC interactions (*e.g*. aldehydes, alcohols) were found to increase upon thermal denaturation of soy proteins and milk proteins (Arai *et al*., 1970; O'Neill & Kinsella, 1988; Ng *et al*., 1989; McNeill & Schmidt, 1993; Hansen & Booker, 1996; Grinberg *et al*., 2002). Others have, however, reported that thermal treatment of soy protein decreased the retention of alcohols (Chung & Villota, 1989). These results show that the effect of heat on VOC retention cannot be easily predicted. The addition of salts may influence VOC retention according to their salting-in and salting-out properties (Nawar 1966; Andriot *et al*., 1999; Guichard & Langourieux, 2000). Chaotropic salts usually reduce VOC retention by destabilising hydrophobic interactions, whereas lyotropic salts usually enhance VOC retention (Lubbers *et al*., 1998). The presence of ethanol could also change protein conformations and hence affect the interaction sites on the protein (Druaux *et al*., 1995; Fischer & Widder, 1997; Lubbers *et al*., 1998). Retention was found to increase with increasing chain-length of VOCs (Damodaran & Kinsella, 1980, 1981a, 1983; O'Neill & Kinsella, 1987b, O'Keefe *et al*., 1991a; Pelletier *et al*., 1998; Andriot *et al*., 2000; Guichard & Langourieux, 2000; Reiners *et al*., 2000). Other factors such as the breaking of disulphide bonds, proteolysis and chemical modifications of the protein's side chains can cause severe structural changes to the protein, and hence affect protein-VOC interactions (O'Neill & Kinsella, 1987a/b, 1988; O'Neill, 1996). Although there are quite some studies on the interactions between VOCs and proteins, the

studies on pea proteins-VOCs interactions are limited (Dumont, 1985; Dumont & Land, 1986). Therefore, additional knowledge on the interactions of VOCs, such as aldehydes and ketones, and pea proteins, would provide a better understanding of the role of flavour compounds in pea protein preparations.

(ii) Protein-saponin interactions

Saponins have also been reported to interact with proteins, such as casein and soy proteins (Potter *et al*., 1993; Ikedo *et al*., 1996; Shimoyamada *et al*., 1998, 2000; Morton & Murray, 2001; Liu *et al*., 2003). Saponins are observed to bind readily to soy protein, whereas their interactions with caseins seem to be less strong (Potter *et al*., 1993). The addition of soyasaponins to bovine serum albumin increased the protein's stability to heat and protease digestion (Ikedo *et al*., 1996). Also, the chymotryptic hydrolysis of soybean protein was suppressed by the addition of soybean saponins (Shimoyamada *et al*., 1998). β-lactoglobulin and α-lactalbumin, however, became more sensitive to both trypsin and chymotrypsin hydrolysis when soybean saponins were added (Shimoyamada *et al*., 2000). Sugarbeet saponins were found to form complexes with proteins at low pH (Morton & Murray, 2001). Results also show that saponins are indeed enriched in protein isolates (Fenwick & Oakenfull, 1981; 1983; Curl *et al*., 1985; Price *et al*., 1985), and may thus influence the taste of the product in which these isolates are used. The interactions with saponins may also influence the interaction of the proteins with added VOCs.

Aims and thesis outline

The aim of this research is to investigate the flavour aspects of peas and its protein fractions, as being potential ingredients for novel protein food products. In order to achieve this, the following objectives were formulated:

(i) To make an inventory of VOCs from pea flour and pea protein preparations.

(ii) To study the interactions of VOCs and pea protein fractions as well as the effect of heating and presence of non-protein components on these interactions.

(iii) To identify and quantify the saponins present in peas, and to characterise their stability and sensory characteristics.

Chapter 2 illustrates a method that was designed for isolating native saponins from peas, and the influence of temperature, pH and solvent quality on the stability of the saponins. The breakdown reaction of DDMP saponin was modelled, and a reaction mechanism of its decomposition was

proposed. Using the method designed for native saponins isolation in chapter 2, the types and amounts of saponin in 16 pea varieties and the difference in bitterness of saponin B and DDMP saponin in relation to their concentrations was investigated in **Chapter 3**. **Chapter 4** gives an overview of the structures of plant saponins that have been isolated and characterised so far, classifies these structures, and investigates the distribution of the main classes of saponins in the plant kingdom. **Chapter 5** gives an overview of the types and amounts of volatile organic compounds released from pea flour, pea protein isolate, and legumin and vicilin preparations. The effect of pH on the release of the volatiles from the pea flour and the effect of protein purification steps were investigated. **Chapter 6** describes the interaction of model volatile organic compounds (aldehydes and ketones of various chain-length) with pea vicilin at various concentrations. Additionally, the effect of heat treatment, as well as the effect of non-protein components (*e.g.* lipids and carbohydrates), on the interactions was investigated. **Chapter 7** gives a general discussion on the results obtained, and relates the importance of flavour compounds of pea ingredients to their use in novel protein food development.

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Chapter 2

Stability of pea DDMP saponin and the mechanism of its decomposition

This Chapter has been submitted to *Food Chemistry* as L. Heng, J.-P. Vincken, K. Hoppe, G.A. van Koningsveld, K. Decroos, H. Gruppen, M.A.J.S. van Boekel, A.G.J. Voragen. Stability of pea DDMP saponin and the mechanism of its decomposition.

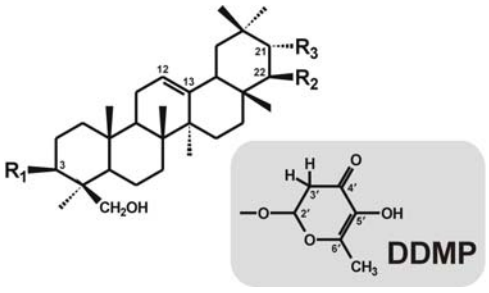
ABSTRACT

DDMP saponin can be converted to saponin B by the loss of its DDMP group (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one). The stability of DDMP saponin from pea was investigated under various conditions (temperature, ethanol concentration, pH). DDMP saponin in water was observed to be unstable at acidic and alkaline pHs, and to have an optimal stability around pH 7. In water, DDMP saponin became unstable at temperatures > 30 ^{o}C. The presence of ethanol, however, had a stabilizing effect on the DDMP group. The loss of the DDMP group at 65 °C could be completely prevented at > 30% (v/v) ethanol. The breakdown reaction of DDMP saponin and the subsequent formation of saponin B was modeled using a multi-response modeling approach and was found to be best described by a first-order reaction. The activation energy was estimated to be 49 kJ/mol, indicating a chemical reaction with moderate temperature dependence. A mechanism of DDMP saponin decomposition is proposed, consisting of a fast protonation or deprotonation followed by a rate-determining step in which maltol is the leaving group.

KEYWORDS: *Green pea(s), DDMP saponin, saponin B, kinetics, ethanol, HPLC, ELSD, modeling*

INTRODUCTION

Saponins are non-volatile, amphiphilic, surface-active triterpene glycosides that occur in a wide variety of legume seeds such as peas, soybeans, lentils and lupins (Lasztity *et al*., 1998). They are known to have a bitter taste (Price *et al*., 1985; Koziol, 1991; Heng *et al*., submitted) and possess health beneficial effects such as lowering cholesterol levels (Milgate & Roberts, 1995). Saponins are generally categorized into 3 main groups, on the basis of their aglycone (soyasapogenol) structures: group A, B and E (**Figure 1**). The aglycones of group A saponins have a hydroxyl group at the C-21 position, whereas those of group B saponins have a hydrogen atom. Group E saponins differ from group B saponins in that their aglycones have a carbonyl group at C-22. Group A saponins are *bisdesmoside* saponins having sugar chains at the C-3 and the C-22 positions of their aglycones (soyasapogenol A), whereas Group B and E saponins are *monodesmoside* saponins having a sugar chain linked to only the C-3 position of their aglycones (soyasapogenol B and soyasapogenol E, respectively) (Shiraiwa *et al*., 1991a/b; Yoshiki *et al*., 1998). Group A saponins may contain acetyl groups attached to the terminal sugar residue of the C22 oligosaccharyl chain (Shiraiwa *et al*., 1991a). Group B saponins may contain a DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) moiety at C-22, which upon heating is released as maltol (Kudou *et al*., 1993). These B saponins are denoted as DDMP saponins.



saponin type	R_1	R_2	R_3
A	$-O-(Gly)_m$	$-O-Gly-[Gly-(OAc)_n]$	–OH
B	$-O-(Gly)_m$	–OH –O–DDMP	–H
E	$-O-(Gly)_m$	=O	–H

Figure 1: Structures of group A, B, E and DDMP saponins.

Saponin B (soyasaponin I) has been reported as the main saponin component in green peas (Price & Fenwick, 1984; Curl *et al*., 1985; Price *et al*., 1985; 1988; Daveby *et al*., 1997; Kinjo *et al*., 1998).

However, it was only until recently that DDMP saponin (soyasaponin βg) was also identified in peas (Daveby *et al*., 1998). DDMP saponin is widely distributed in legumes (Yoshiki *et al*., 1994) and is the predominating saponin in 16 different pea varieties (Heng *et al*., submitted) and in soybean (Kudou *et al*., 1992; 1993; Hu *et al*., 2002; Lin & Wang, 2004). Saponin B is the major saponin in processed soy products and heated pea products, presumably due to the conversion of DDMP saponin to saponin B during extraction and processing (Berhow *et al*., 2002; Hu *et al*., 2002; Heng *et al*., submitted). In addition, reports have shown that DDMP saponin is converted to saponin B in acidic or basic solutions (Massiot *et al*., 1996; Okubo & Yoshiki, 1996). These results indicate that DDMP saponin is unstable under conditions commonly applied during food processing, but detailed studies mapping these conditions are lacking. A systematic study of DDMP saponin stability will provide information, not only on the effect of extraction and processing, but may also provide tools on the possible removal or masking of the undesirable bitterness of saponins in pea product (Heng *et al*., submitted). The aim of our research was to study the stability of DDMP saponin at various conditions of temperature, ethanol concentration and pH.

MATERIALS AND METHODS

Saponin extraction

Saponins were extracted from peas (*Pisum* s*ativum, Solara spp.*) obtained from Cebeco (Vlijmen, The Netherlands). The extraction procedure is illustrated in **Figure 2**. The peas were milled in a commercial blender (Waring, New Hartford, Connecticut, U.S.A.) in the ratio 1:1 (w/w) with dry ice. Pea flour was defatted by hexane (Super gradient, Lab-scan, Dublin, Ireland) refluxing for 6 h and subsequently the pea flour was air-dried in a fume hood overnight. Defatted pea flour (1 g) was extracted with 70% (v/v) ethanol (100 mL) for 1 hour at 25 ^{o}C with constant shaking at 200 rpm in an incubator shaker (Innova 4000, New Brunswick Scientific, Nijmegen, The Netherlands). The crude extract was filtered through an ashless filter paper (White band 589^{2}, 110 mm, Schleicher & Schuell, Dassel, Germany). The ethanol from the clear filtrate was evaporated under vacuum at 27 ^{o}C. This evaporation step was performed in less than 15 min using a 1 L round-bottom flask. The removal of ethanol made the extract turbid, and hence the resulting extract (~30 mL) was made up to 40 mL with distilled water and was centrifuged (36,000 x *g*; 10 min; 10 ^{o}C). The supernatant obtained was passed through a *Sep-Pak* C18 solid phase extraction column (400 mg, Waters Plus tC18 cartridge, 37-55 μm, Waters Etten-Leur, The Netherlands), which was subsequently rinsed with 15 mL water to remove unbound material. The bound compounds were eluted with 10 mL of 100% (v/v) methanol (HPLC grade; Lab-Scan, Dublin, Ireland) and air-dried. The air-dried saponin sample was solubilized in 1 mL of 50% (v/v) ethanol and centrifuged (36,000 x *g*; 10 min) before HPLC analysis. For stability tests, the extraction was upscaled to 5 g of pea flour in 500 mL of 70% (v/v) ethanol, and a 5 g Waters Plus tC18 column, 37-55 μm (Waters Etten-Leur, The Netherlands) was used.

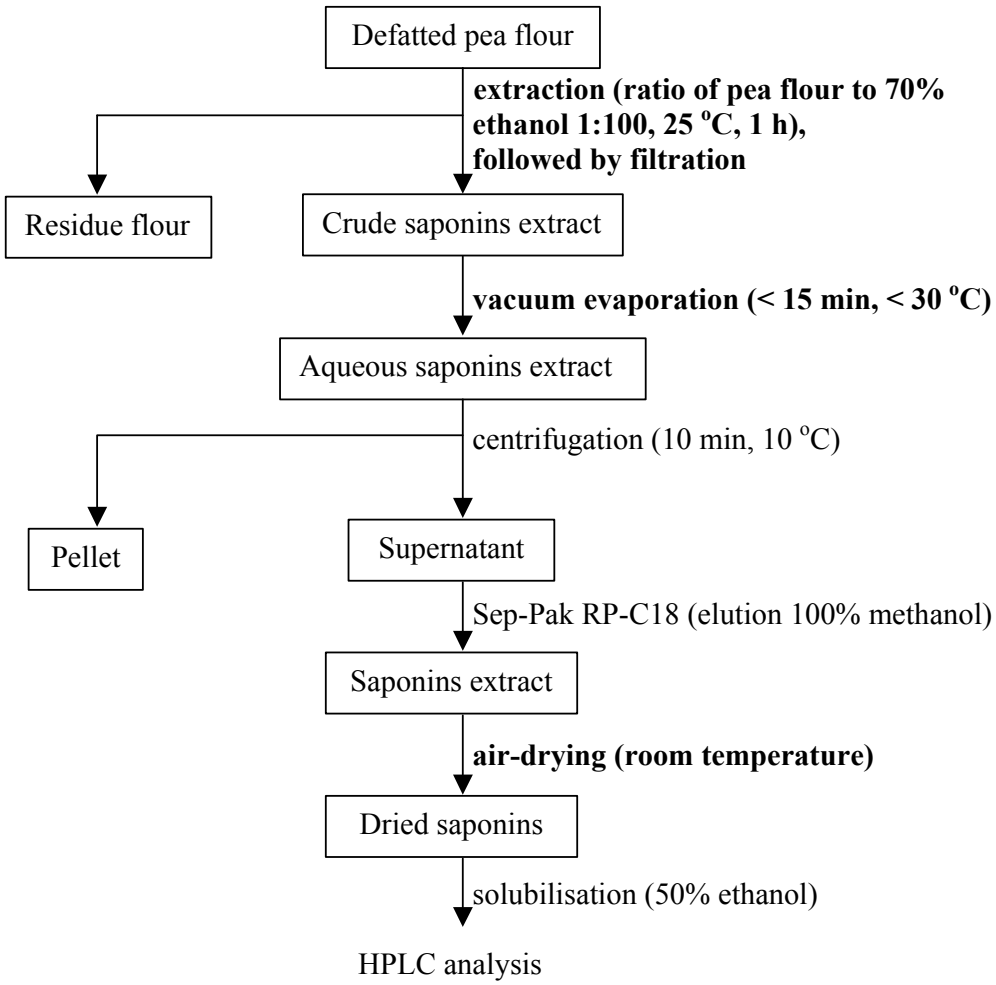


Figure 2: Optimized extraction protocol for obtaining native saponins from pea flour. Critical steps in the procedure are in bold.

High performance liquid chromatography – Mass Spectrometry (HPLC-MS) analysis

Reversed-phase high performance liquid chromatography (RP-HPLC) was used for the analysis of pea saponins. Evaporative light scattering detection (ELSD) was used for detection and ion trap electrospray mass spectrometry (MS) was used for component identification. A SpectraSYSTEM HPLC (Thermo Separation Products, Fremont, CA) coupled to a Sedex 55 ELS detector (S.E.D.E.R.E., Alfortville, France) was used. Separation was performed using an Aquasil reversed-phase C18 column (4.6 x 150 mm, 3 μm) (Thermo Hypersil, Bellefonte, P.A., U.S.A.). The solvents used were water:acetic acid (100:0.001, v/v) (A) and acetonitrile:acetic acid (100:0.001, v/v) (B). The gradient used was as follows: 0→8 min, 40→50% B; 8→10 min, 50→100% B; 10→15 min, 100% B (isocratic); 15→20 min, 40% B (isocratic). Samples of 20 μL were injected and a flow rate

of 1 mL/min was used. The eluate from the column was split into 3 directions: 100 µL/min to the ELSD, 50 µL/min to the LCQ Ion-trap MS (Thermo Finnigan, San Jose, CA) and 850 µL/min to the waste. The ELSD was set at 40 ^{o}C at an air pressure of 2.3 bars and a sensitivity of 12. Quantification of DDMP and B saponins was done by means of the response factors determined by Decroos *et al*. (2005). The decrease in DDMP saponin concentration was obtained by integrating the area of its peak (after heat treatment) in the HPLC-chromatogram, whereas the increase in saponin B concentration was obtained by subtracting the initial saponin B area from the area of the saponin B peak obtained after heat treatment. MS analysis was performed in the positive ion mode using a spray voltage of 5.5 kV, a capillary voltage of 15 V and a capillary temperature of 200 ^{o}C. A full scan mass spectrum over a *m/z* (mass to charge ratio) range of 150-1500 was obtained. The mass spectra were recorded and analyzed with the use of *Xcalibur* software. The *m/z* ratio of the molecular ions $[M+H]^{+}$ in the mass spectra of the peaks of DDMP saponin and saponin B were 1069 and 943, respectively.

Stability tests

(i) Effect of temperature

Saponin samples of 1 mg/mL were prepared in water (pH 6.7). Temperatures of 40, 50, 60, 65, 75 and 90 ^{o}C were chosen. At each temperature, the samples were heated for 15, 30, 45, 60 and 75 min with constant shaking at 400 rpm in an incubator shaker (Eppendorf AG, Thermomixer comfort, Hamburg, Germany). The samples were cooled to room temperature before an equal volume of absolute ethanol was added. The samples were centrifuged at 24,700 x *g* at 10 ^{o}C for 5 min, and 20 µL of the supernatant was injected onto the HPLC column for analysis as described above. All experiments were performed in triplicate.

(ii) Effect of ethanol

Saponin samples of 1 mg/mL were prepared in aqueous ethanol containing: 0, 5, 10, 15, 20, 30, 50 and 60% (v/v) ethanol. The samples were heated at 65 ^{o}C for 15, 30, 45, 60, 75 and 90 min with constant shaking at 400 rpm in an incubator shaker (Eppendorf AG, Thermomixer comfort, Hamburg, Germany). The samples were cooled to room temperature, centrifuged at 24,700 x *g* at 10 ^{o}C for 5 min, and 20 µL of the supernatant was injected onto the HPLC column for analysis. All experiments were performed in triplicate.

(iii) Effect of pH

Saponin samples of 1 mg/mL were prepared in solutions of various pHs (2 to 10). Solutions at pH 2 to 6 were prepared in water containing HCl in molarities of 0.00125 to 12.5 mM. Solutions at pH 8 to 10 were prepared in water containing NaOH in molarities of 0.00125 to 1.25 mM. All solutions were stored under nitrogen. 20 µL of a saponin stock solution of 5 mg/mL was added to 80 µL of the solutions of various pH. The pH of the samples was measured and the samples were flushed with nitrogen, followed by incubation at 35 ^{o}C for 20 h. After incubation, the pH of the samples was measured again, followed by adjustment of the pH of the samples to pH 7 with HCl or NaOH, with only negligible change in sample volume. The samples were centrifuged at 24,700 x *g* at 10 ^{o}C for 5 min and 20 µL of the supernatant was injected onto the HPLC column for analysis. All experiments were performed in triplicate.

Modelling of the thermal stability of DDMP saponin

A first-order kinetic model was applied for characterizing DDMP saponin stability according to the following reaction:

$$\text{DDMP} \xrightarrow{k} \text{B}$$

The concentrations of both DDMP saponin and saponin B (as a function of time) were taken into account in the modeling, using the so-called multi-response modeling approach (van Boekel, 1996). The initial concentrations of DDMP saponin and saponin B were measured, and the rate constant *k*, was estimated. Upon starting the experiments, both DDMP and saponin B were present, and subsequent heating caused a breakdown of DDMP. Because of the relatively large differences in the initial concentrations, it was decided to model the initial concentrations rather than fixing them. The parameters were estimated by nonlinear regression in order to avoid undesirable effects of logarithmic transformations (van Boekel, 1996). The software used was Athena Visual Workbench, a program designed for multi-response modeling (www.athenavisual.com). The activation energy was derived from the temperature dependence of the rate constants using Arrhenius law.

RESULTS

Optimization of extraction conditions

The DDMP saponin that is present in peas is heat labile (Daveby *et al*., 1998) and can be converted to its corresponding saponin B (Daveby *et al*., 1998; Berhow *et al*., 2002; Heng *et al.*, submitted). As the extraction conditions (temperature, time) seem to determine the DDMP saponin content (Daveby *et al*., 1998), the extraction temperature and time were the primary parameters to be taken into consideration when designing an extraction method. The extraction procedure described in the Materials and Methods section and as shown in **Figure 2** was obtained after careful optimization. Temperatures during extraction and drying of 20 ^{o}C, 30 ^{o}C and 40 ^{o}C, extraction times of 1 h, 2 h, 3 h, 4 h and 24 h, and extraction volumes of 100 mL and 300 mL per gram of defatted pea flour were investigated. DDMP saponin was extracted as the major saponin in green peas at all extraction conditions with a small amount of saponin B (results not shown). DDMP saponin was observed to remain stable at temperatures up to 30 ^{o}C, whereas a small decrease in stability was observed at 40 ^{o}C. Lin & Wang (2004) also observed that DDMP saponin remained relatively stable at temperatures ≤ 30 ^{o}C. Extraction times longer than 1 h or extraction volumes larger than 100 ml per gram of pea flour did not affect the amount of saponins extracted. Also, the ratio of saponin B to DDMP saponin (1:4) in the extract remained constant even after 24 h of extraction, provided that the temperature was kept below 30 ^{o}C, and the evaporation step before solid phase extraction was performed in less than 15 min. The removal of ethanol resulted in a turbid extract, but the pellet obtained after centrifugation showed no traces of saponins.

Through systematic investigation of these parameters, the optimal conditions for the extraction of saponins from peas were found: the saponins from one gram of pea flour were extracted using 100 mL of 70% (v/v) aqueous ethanol during 1 h at 25 ^{o}C (Materials and Methods). With the use of this isolation method, DDMP saponin was obtained as the major saponin present in green peas and it was used for the systematic stability study concerning the effects of temperature, pH and ethanol concentration on the concentration of DDMP saponin.

Effect of temperature on DDMP saponin

The concentration of DDMP saponin as a function of time at various temperatures is shown in **Figure 3**. The concentration of DDMP saponin remained stable in water at temperatures ≤ 30 ^{o}C for a period of at least 75 min. Upon increasing the temperature, a small decrease in DDMP saponin

concentration with time was observed at 40 °C. Above 40 °C, the decrease in concentration of DDMP saponin became more pronounced, with a 50% and a 75% decrease in DDMP saponin concentration after 75 min at 60 °C and 75 °C, respectively. At 90 °C, the decrease of DDMP saponin concentration was even faster than at 75 °C, and a decrease to 5% was reached within 75 min. At 50 °C and 65 °C, similar trends were observed (results not shown).

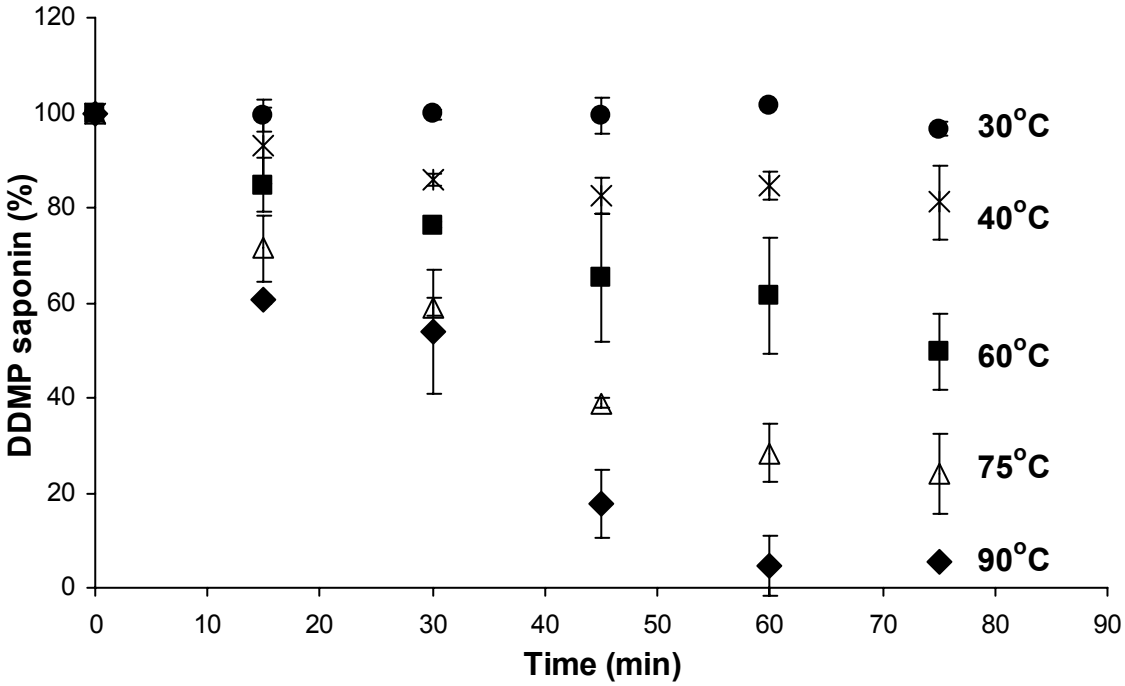


Figure 3: Effect of temperature on the DDMP saponin concentration as a function of time.

Modeling of the (thermal) stability of DDMP saponin

With the results obtained from the thermal stability studies, a model was applied for analyzing the DDMP saponin degradation kinetics and to obtain the activation energy (E_a) of the reaction. **Figure 4** shows the course of DDMP saponin and saponin B concentrations as a function of time, and the fit of the applied model, when a temperature of 65 °C is applied. The breakdown of DDMP saponin was, at all temperatures, observed to result in the parallel formation of saponin B. Although some individual results showed a relatively high degree of variation, the molar sum of DDMP saponin and saponin B was more or less constant. The results modeled indicate that the breakdown reaction

of DDMP saponin ($\mathrm{DDMP} \xrightarrow{k} \mathrm{B}$) follows a first-order reaction, and that there are no other parallel or consecutive reactions of quantitative importance.

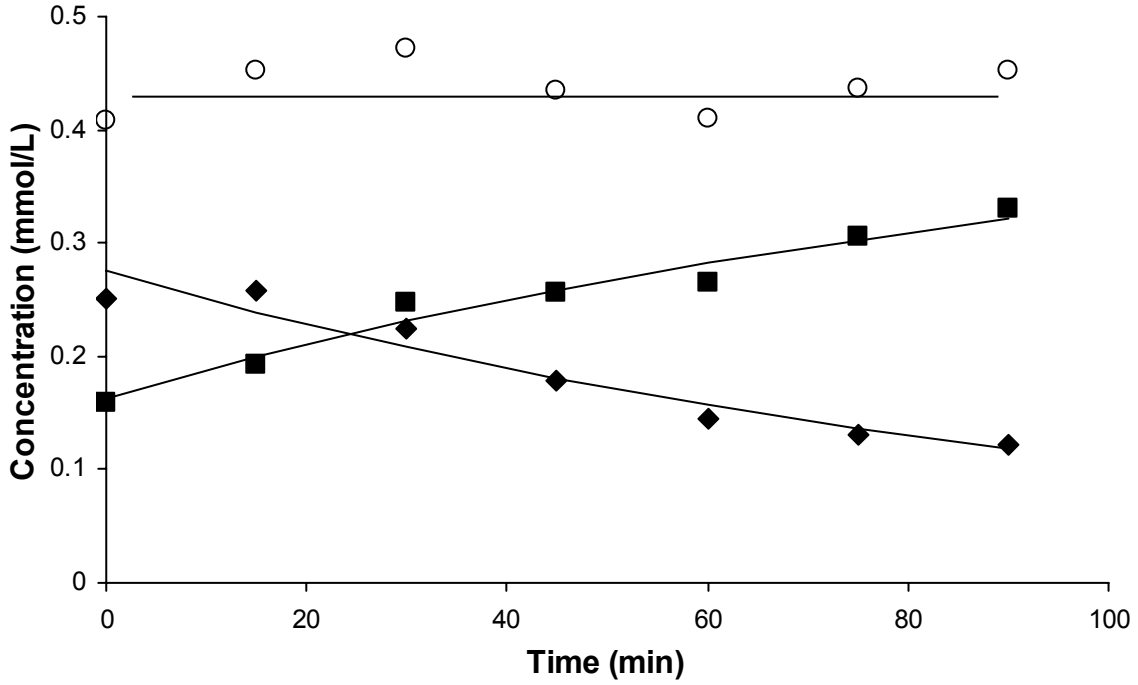


Figure 4: Degradation of DDMP saponin (♦) and formation of saponin B (■) after heating at 65 ºC. The molar sum of DDMP saponin and saponin B is also indicated (o). The solid lines represent the model fit of the proposed first order reaction.

Table 1 shows the resulting estimates of the observed rate constant k (± 95% confidence intervals) for the degradation of DDMP saponin and the formation of saponin B at various temperatures.

The E_a of the reaction was derived from the temperature dependence of the rate constant using Arrhenius law:

$$k = k_0 \exp(-\frac{E_a}{RT})$$

In this equation, k_0 represents the pre-exponential factor, E_a the activation energy, R the gas constant, and T the absolute temperature. In order to avoid statistical problems with nonlinear regression, due to a strong correlation between the parameters k_0 and E_a, this equation was reparameterized, as described by van Boekel (1996), to:

$$k = k_{ref} \exp(-\frac{E_a}{R}(\frac{1}{T} - \frac{1}{T_{ref}}))$$

In this equation, T_{ref} and k_{ref} are the reference temperature and the rate constant at the reference temperature, respectively. The reference temperature was chosen as the average of all the temperatures investigated; in this case 63.3 °C. E_a and k_{ref} were estimated using weighted nonlinear regression. The weights were derived from the confidence intervals of the rate constants shown in **Table 1**. The estimated E_a was 49 ± 12 kJ/mol and the reference rate constant, k_{ref} was 0.0053 ± 0.0005 min^{-1} (± 95% confidence interval).

Table 1: The resulting estimates of the rate constant k ± 95% confidence intervals for the degradation of DDMP saponin and the formation of saponin B at various temperatures

Temperature (°C)	Experiment no.	k (min^{-1})
40	1	0.0012 ± 0.0006
	2	0.0009 ± 0.0004
	3	0.0015 ± 0.0007
50	1	0.0028 ± 0.0007
	2	0.0014 ± 0.0006
60	1	0.0047 ± 0.0003
	2	0.0029 ± 0.0006
	3	0.0063 ± 0.0013
65	1	0.0067 ± 0.0009
	2	0.0056 ± 0.0003
	3	0.0094 ± 0.0008
	4	0.0067 ± 0.0009
	5	0.0056 ± 0.0003
75	1	0.0100 ± 0.0009
	2	0.0076 ± 0.0015
	3	0.0130 ± 0.0043
90	1	0.0168 ± 0.0049
	2	0.0157 ± 0.0027

Figure 5 shows the Arrhenius plot obtained using the estimates derived from the model via nonlinear regression. The Arrhenius equation was applied over the whole temperature range studied. The Arrhenius plot does not appear to be completely linear: *i.e*. at higher temperatures the temperature dependence seems to be weaker than that at lower temperatures. However, the variation in the data is too high to draw valid conclusions.

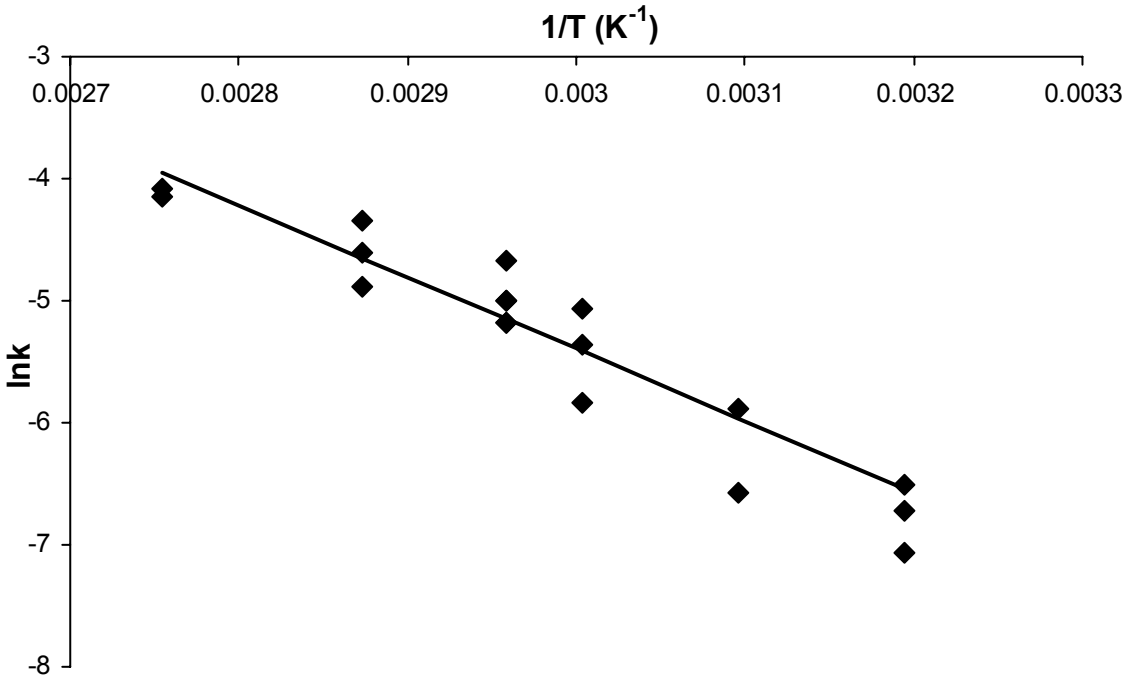


Figure 5: Arrhenius plot for the rate constant describing the breakdown of DDMP saponin and formation of saponin B. The solid line is drawn using the parameter estimates obtained via nonlinear regression (cf. E_a = 49 ± 12 kJ/mol; k_{ref} = 0.0053 ± 0.0005 min^{-1}; ± 95% confidence interval).

Effect of ethanol

The effect of ethanol concentration on the stability of DDMP saponin treated at 65 ^oC for 90 min is shown in **Figure 6**. The concentration of DDMP saponin at 65 ^oC remained stable up to 90 min at ethanol concentrations of 30% (v/v) ethanol. When the ethanol concentration was decreased to 10% (v/v), the concentration of DDMP saponin decreased with time to approximately 60% of the initial concentration after 90 min. In the absence of ethanol, however, a much faster decrease in the concentration of DDMP saponin was observed. The effects of ethanol concentrations of 5%, 15%,

20%, 50% and 60% (v/v) on the stability of DDMP saponin were also studied (results not shown). At 5%, 15% and 20% (v/v) ethanol, DDMP saponin concentration showed a similar trend with time as at 10% (v/v) ethanol. At 50% and 60% (v/v) ethanol, the DDMP saponin concentration at 65 ^{o}C remained constant.

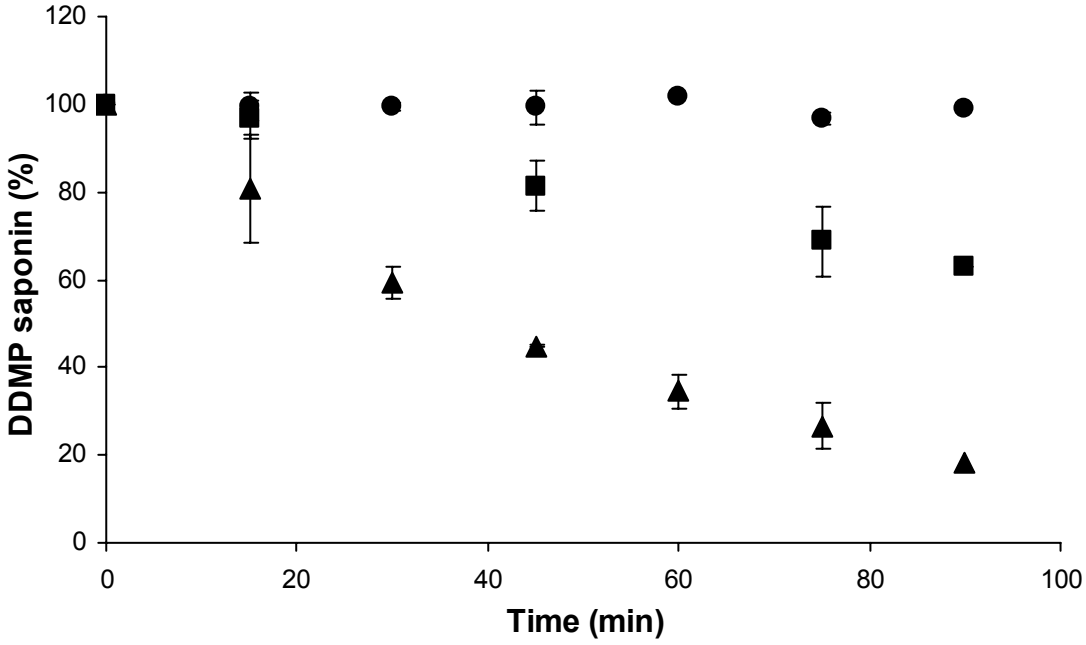


Figure 6: DDMP saponin decomposition as a function of time (65 ^{o}C) at various ethanol concentrations of 0% (▲), 10% (■) and 30% (●).

Effect of pH

The effect of pH on the stability of DDMP saponin, upon incubation at 35 ^{o}C for 20 h, is shown in **Figure 7**. It can be seen that DDMP saponin is stable only within a relatively narrow pH range with an optimal stability around pH 7. At acidic or basic pH, the stability of DDMP saponin decreased drastically. A change of pH by one unit may already result in a 20-60% decrease in DDMP saponin concentration under the conditions used. The degradation of DDMP saponin resulted in a proportional increase in saponin B.

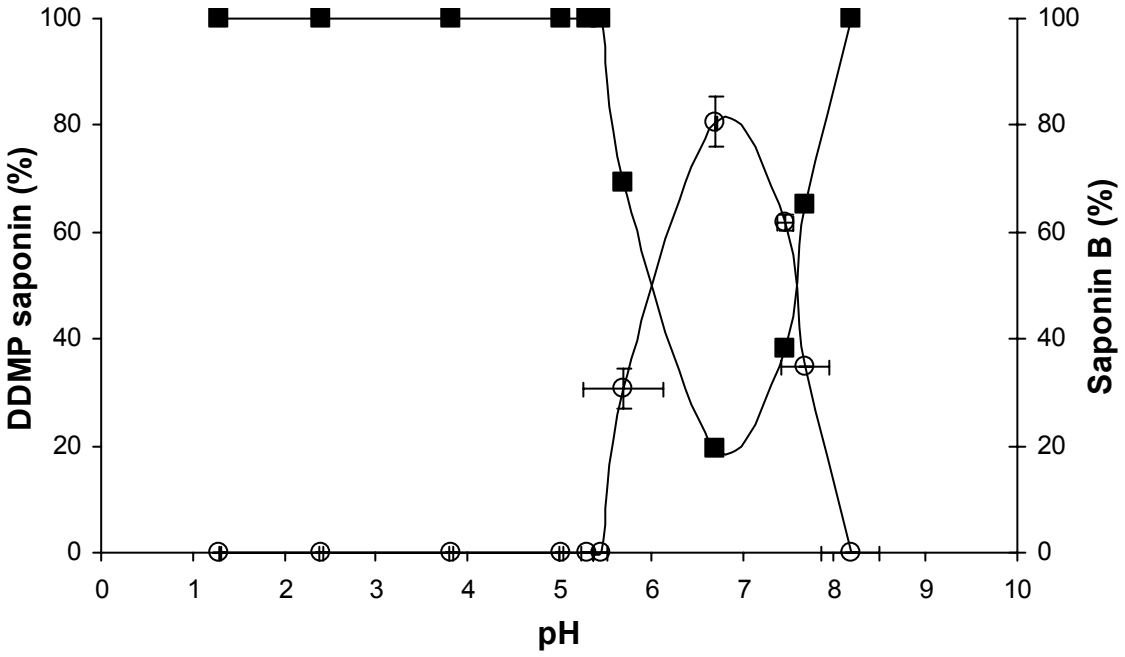


Figure 7: Effect of pH on DDMP saponin content after (o) incubation at 35 ^{o}C for 20 h. The saponin B content after incubation is also shown (■).

DISCUSSION

Effect of temperature

DDMP saponin was found to be the major saponin present in pea, but it was also found to be easily converted to saponin B. DDMP saponin was observed to be stable when extraction temperatures were kept at ≤ 30 ^{o}C (Daveby *et al*., 1998; Hu *et al*., 2002; Lin & Wang, 2004). Our results show that the time of exposure has a profound effect on DDMP saponin degradation but only at temperatures ≥ 40 ^{o}C. The estimated E_a (49 ± 12 kJ/mol) for the first order DDMP saponin degradation reaction is relatively low for a chemical reaction, which normally has an E_a between 50 and 100 kJ/mol. This low E_a explains why the decomposition of DDMP saponin proceeds at relatively low temperatures during extraction and incubation.

Proposed mechanism for decomposition of DDMP saponin

A reaction mechanism for the decomposition of DDMP saponin is proposed in **Figure 8**. The bell-shaped DDMP saponin stability profile in **Figure 7** showed that the decomposition reaction can be catalyzed by either acid or base. Under acidic conditions (**Figure 7**, top reaction), the C22-O-C2′ ether linkage (see **Figure 1**) is probably protonated. Such a proton transfer reaction generally proceeds very rapidly, and is diffusion-controlled. This rapid pre-equilibrium step is followed by a much slower reaction, in which the DDMP group dissociates from the C-22 position of the saponin B aglycone (denoted as 'R'), leading to the formation of the intermediate product I_A. This rate-determining step is in principle a mono-molecular process, which is consistent with our results indicating that the breakdown of DDMP saponin complies with first-order kinetics. Subsequently, I_A is converted to the stable product, maltol (compound II), which is resonance stabilized (note the aromatic π-system in compound III). Under alkaline conditions, one of the acidic protons attached to C3′ is presumably abstracted (fast), followed by the formation of intermediate I_B (slow), which is subsequently converted to the same stable product, maltol.

Similar to our observations, DDMP saponin has been reported to be converted to saponin B in both acidic and basic solutions (Massiot *et al*., 1996). However, in contrast to this, Okubo and Yoshiki (1996) found that DDMP saponin was relatively stable in acidic solutions, but was easily hydrolyzed in alkaline solutions. It has been suggested by others (Okubo & Yoshiki, 1996) that the presence of metal ions (such as Fe^{3+}) may cause hydrolysis of DDMP saponins. In water, these

metal ions may form relatively strong acids, *e.g*. the pK_a of $Fe(H_2O)_6^{3+}$ is 2.2 (Li *et al*., 1996). The bell-shaped pH - [DDMP saponin] plot (**Figure 7**) shows that a slight decrease of pH already causes a considerable decrease in DDMP saponin stability, and hence, the presence of small amounts of Fe^{3+} may already result in considerable losses of DDMP saponin. However, it cannot be excluded that Fe^{3+} directly binds to the DDMP saponin, and in this way catalyses the hydrolysis of the compound.

Ethanol as a stabilizer

The effect of ethanol became apparent in the sample clean-up step during purification of the saponins. The use of reversed-phase solid phase extraction columns, such as *Sep-Pak*, necessitated the removal of ethanol from the extract. It was observed that during the evaporation of ethanol, a decrease in the concentration of DDMP saponin occurred. This effect became more pronounced at longer evaporation times. Together with the results in **Figure 6**, this indicates that the presence of ethanol was essential at elevated temperatures to prevent decomposition of DDMP saponin.

The proposed mechanism (**Figure 8**) also provides an explanation for the observed protective effect of ethanol in the decomposition reaction. Water has a much higher relative dielectric constant (82) than ethanol (24) (Shriver *et al*., 1990), which means that water is a much more favorable environment for the formation of charged intermediates (I_A, I_B and III) (**Figure 8**) than ethanol or ethanol/water mixtures. By lowering the dielectric constant, addition of ethanol may thus shift the equilibria in such a way that DDMP saponin is stabilized. It seems that the decomposition reaction does not proceed further at ethanol concentrations of > 30% (**Figure 6**), *i.e*. below a relative dielectric constant of 60 (20 ^{o}C) (Hong *et al*., 1999). Therefore, the evaporation time and temperature during DDMP saponin purification should be limited, so as to reduce the time of exposure of DDMP saponin to a too aqueous environment at elevated temperatures. Oleszek *et al*. (1990) and Hu *et al*. (2002) also found that saponin samples remained relatively stable in methanol at temperatures < 0 ^{o}C. However, contradictory to these reports and our findings, Massiot *et al*. (1996) and Daveby *et al*. (1998) reported that DDMP saponin could decompose to saponin B in alcoholic solutions.

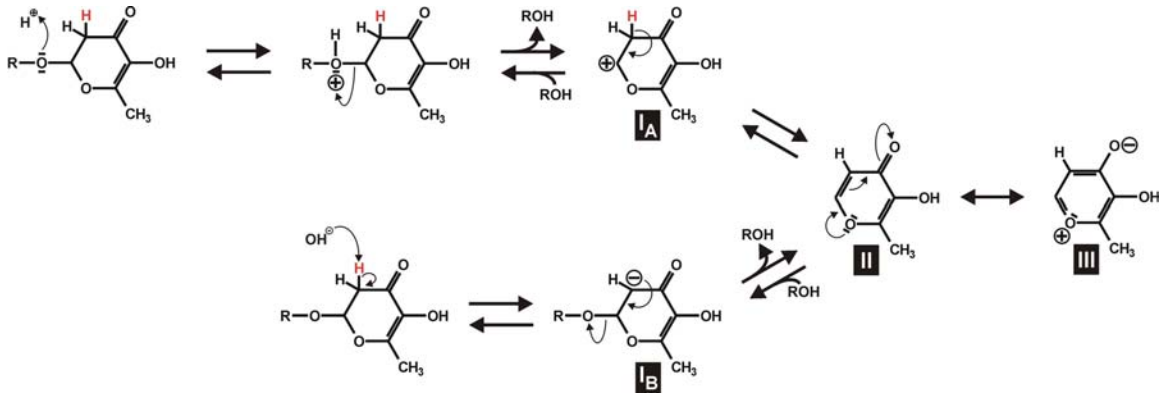


Figure 8: Mechanism for DDMP saponin decomposition at acidic and alkaline pH. R is the saponin B aglycone as in Figure 1. I_A and I_B are intermediate products catalyzed by acid and base, respectively.

This study has shown that the decomposition of DDMP saponin is an acid/base-catalyzed, pseudo first-order reaction with a relatively low activation energy. The reaction can be inhibited by lowering the temperature and the dielectric constant of the solvent, and maintaining a neutral pH. These factors should be considered when performing quantitative studies on native saponins in plant extracts.

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Chapter 3

Bitterness of saponins and their contents in peas

This Chapter has been submitted to *J. Sci. Food Agric.* as L. Heng, J.-P. Vincken, G.A. van Koningsveld, A. Legger, H. Gruppen, M.A.J.S. van Boekel, J.P. Roozen, A.G.J. Voragen. Bitterness of saponin and their contents in peas.

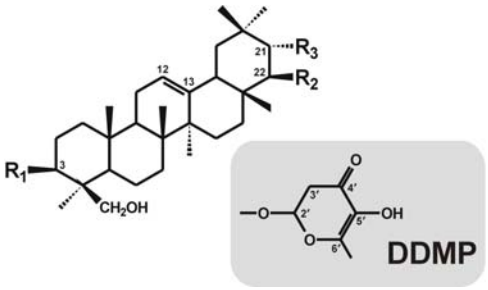
ABTRACT

The bitterness of a saponin mixture (containing saponin B and DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) saponin in a ratio of 1:4) and saponin B obtained from peas, were established by a trained panel using line scaling. Both saponins were found to be bitter. However, the saponin mixture, and hence DDMP saponin, was found to be significantly more bitter than saponin B. The bitterness perceived correlates with the saponin concentration. In addition to saponin bitterness, the saponin content and composition of 16 pea varieties was also investigated. In 2 varieties, DDMP saponin was the only saponin present, whereas in the rest of the varieties DDMP saponin was more abundant than saponin B. The pea varieties investigated differed significantly in saponin content. The amounts of DDMP saponin ranged from 0.7-1.5 g/kg (dry matter), whereas those of saponin B ranged from 0-0.4 g/kg. As saponins are bitter, pea varieties with lower saponin contents would be preferred for use in food production.

KEYWORDS: *Peas, DDMP saponin, saponin B, bitterness, sensory, varieties*

INTRODUCTION

Saponins are non-volatile, amphiphilic, surface-active triterpene glycosides that occur in a wide variety of legume seeds such as peas, soybeans, lentils and lupins (Lasztity *et al*., 1998). Saponins are generally categorised into 3 main groups, on the basis of their aglycone (soyasapogenol) structures: group A, B and E (**Figure 1**). The aglycones of group A saponins have a hydroxyl group at the C-21 position whereas those of group B saponins have a hydrogen atom. Group E saponins differ from group B saponins in that their aglycones have a carbonyl group at C-22. Group A saponins are *bisdesmoside* saponins having sugar chains at the C-3 and the C-22 positions of their aglycones (soyasapogenol A), whereas Group B and E saponins are *monodesmoside* saponins having a single sugar chain linked to the C-3 position of their aglycones (soyasapogenol B and soyasapogenol E, respectively) (Shiraiwa *et al*., 1991a/b). Group A saponins may contain acetyl groups attached to the terminal sugar residue of the oligosaccharyl chain, which is linked to the C-22 position of the aglycon (Shiraiwa *et al*., 1991a). Group B saponins may contain a DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) moiety, linked to the C-22 hydroxyl group, which upon heating, is released as maltol (Kudou *et al*., 1993). These B saponins are denoted as DDMP saponins.



saponin type	R_1	R_2	R_3
A	–O–$(Gly)_m$	–O–Gly–[Gly–$(OAc)_n$]	–OH
B	–O–$(Gly)_m$	–OH –O–DDMP	–H
E	–O–$(Gly)_m$	=O	–H

Figure 1: Structures of group A, B, E and DDMP saponins.

A large number of different saponins may also occur within a single plant species (Price *et al*., 1988). In legumes, varietal differences with respect to saponin content and composition have also been observed, *e.g*. in soybeans (Shiraiwa *et al*., 1991c), navy beans (Fenwick & Oakenfull, 1983), moth beans (Khokhar and Chauhan, 1986), lentils (Ruiz *et al*., 1997) and pigeon peas (Duhan *et al*., 2001). Saponin contents in the range of 0.8-2.5 g/kg have been found for different pea cultivars

(Bishnoi & Khetarpaul, 1994; Daveby *et al*., 1997). Detailed studies on the composition and content of saponins in peas are scarce. It has been reported that group B saponins are the only type of saponin present (Price & Fenwick, 1984; Curl *et al*., 1985; Price *et al*., 1985; 1986; 1988; Daveby *et al*., 1997; Kinjo *et al*., 1998), whereas others have reported also the presence of DDMP saponins (Daveby *et al*., 1998). These results indicate that there are likely two predominant saponin types in pea, but it remains unclear which type predominates. As DDMP saponin is easily converted to saponin B, it is possible that DDMP saponin is in fact the native saponin present in pea. The different findings on the presence and contents of DDMP saponins in pea may be largely due to different methods of extraction and quantification (Heng *et al*., submitted).

The bitterness of peas, as well as that of soybeans, has been ascribed to the presence of saponins (Price & Fenwick, 1984; Price *et al*., 1985; Okubo *et al*., 1992). Protein-rich fractions from pea were given high sensory ratings on bitterness and astringency, presumably due to the presence of high amounts of saponins (Price *et al*., 1985). As there is an increasing interest in the use of pea proteins in food products, it is important that the taste and flavour of these protein foods are appealing to consumers. However, detailed studies on the relationship between saponin content and bitterness have not been performed for pea products. It is also not known if and how bitterness is related to the type of saponin present. Therefore, the aim of the present study was to identify and quantify the type of saponins present in different pea varieties and to evaluate their bitterness.

MATERIALS AND METHODS

Materials

All pea seeds were obtained from Cebeco Zaden B.V. (Lelystad, The Netherlands) except 3 pea lines (*Pisum arvense*, *Pisum elatius Marbre, Pisum elatius 1140175*), which were obtained from the Center for Genetic Resources (CGN, Wageningen, The Netherlands). The pea varieties tested and their characteristics are summarized in **Table 1.** Caffeine (99% purity), equilenin and maltol were obtained from Sigma-Aldrich Chemie GmbH, Steinheim, Germany. Quinine sulphate dihydrate (99% purity) was obtained from Janssen Chimica, Geel, Belgium.

Table 1: Seed characteristics of 16 pea varieties

Line	Name	Color [1]	Shape [2]
1	KPMR 146 India	Yel	F
2	Pisum elatius, 1140175 (CGN	Br-Grey-sp	R
3	Cisca	Gr	W
4	FAL 49110 Mongolie	Yel	R
5	CEB 1475.1	Yel	R
6	Baccara	Yel	R-D
7	CEB 1466	Yel	R
8	Pisum arvense (CGN 10193)	Br-Gr-sp	F
9	Pisum elatius, Marbre (CGN 03351)	Br-Gr-sp	F
10	Courier	Br-sp	F
11	6 S 41.4	Gr	F
12	Solara	Gr	R
13	NGB 102149 Iran	Br-Gr-sp	F
14	Solido	Br	W
15	Supra	Gr	W
16	NGB 101293 Jordaan	Gr-sp	F

[1] Seed colour abbreviations: Yel = yellow, Gr = green, Br = brown, sp = spotted

[2] Seed shape abbreviations: W = wrinkled, R = round, R-D= round with dimples, F = flat

Saponin extraction

The peas were milled in a commercial blender (Waring, New Hartford, Connecticut, U.S.A.) in a ratio 1:1 (w/w) with dry ice. Pea flour was defatted by hexane (Super gradient, Lab-scan, Dublin, Ireland) refluxing for 6 h and subsequently the pea flour was air-dried in a fume hood overnight. Defatted pea flour (1 g) was extracted with 70% (v/v) ethanol (100 mL) for 1 h at 25 ^{o}C with constant shaking at 200 rpm in an incubator shaker (Innova 4000, New Brunswick Scientific, Nijmegen, The Netherlands). Prior to extraction, 100 ppm of an internal standard, equilenin (an estrogen-like steroid, 3-hydroxyestra-1,3,5,7,9-pentaen-17-one) was added. The crude extract was filtered through an ashless filter paper (White band 589^{2}, 110 mm, Schleicher & Schuell, Dassel, Germany). The ethanol from the clear filtrate was removed by evaporation under vacuum at 27 ^{o}C. This evaporation step was performed in less than 15 min using a 1 L round-bottom flask. The removal of ethanol made the extract turbid, and hence the resulting extract (~30 mL) was made up to 40 mL with distilled water and was centrifuged (36,000 x *g*; 10 min; 10 ^{o}C). Analysis of the resulting pellet showed no traces of saponin. The supernatant obtained was passed through a *Sep-Pak* C18 solid phase extraction column (Waters Plus tC18 cartridge, 37-55 μm, Waters Etten-Leur, The Netherlands), which was subsequently rinsed with 15 mL water to remove unbound materials. The bound compounds were eluted with 10 mL of 100% (v/v) methanol (HPLC grade; Lab-Scan, Dublin, Ireland) and air-dried. The air-dried saponin sample was dissolved in 1 mL of 50% (v/v) ethanol and centrifuged at 36,000 x *g* for 10 min, before HPLC analysis.

Determination of saponin content and composition

Reversed-phase high performance liquid chromatography (RP-HPLC) in combination with evaporative light scattering detection (ELSD) was used for analysing pea saponins. A SpectraSYSTEM HPLC (Thermo Separation Products, Fremont, CA) coupled to an Alltech ELSD 2000 detector (Deerfield, U.S.A.) was used. Separation was performed using an Aquasil reversed-phase C18 column (4.6 x 150 mm, 3 μm) (Thermo Hypersil, Bellefonte, P.A., U.S.A.). The solvents used were water:acetic acid (100:0.001, v/v) (A) and acetonitrile:acetic acid (100:0.001, v/v) (B). The linear gradient used was as follows: 0→8 min, 40→50% B; 8→10 min, 50→100% B; 10→15 min, 100% B (isocratic); 15→20 min, 40% B (isocratic). Samples of 20 μL were injected and a flow rate of 1 mL/min was used. The eluate from the column was split into 3 directions: 100 μL/min to the ELSD, 50 μL/min to the LCQ Ion-trap MS (Thermo Finnigan, San Jose, CA) and 850 μL/min to the waste. The ELSD was set at 40 ^{o}C at a gas flow of 1.5 mL/min and a sensitivity of

16. Components were identified by mass spectrometry according to Heng *et al*. (submitted). The *m/z* ratio of the molecular ions $[M+H]^+$ in the mass spectra of the peaks of DDMP saponin and saponin B were 1069 and 943, respectively. Quantification of DDMP and B saponins was done using response factors obtained from calibration of ELSD according to Decroos *et al*. (2005).

Sensory evaluation of the bitterness of saponin extracts from peas

(i) Selection of panellists

Representative standard bitter compounds, caffeine and quinine sulphate dihydrate were used for the selection procedure. From both compounds, 7 concentrations were prepared by successive dilution with tap water. The concentrations were based on the threshold values reported in literature (Mojet *et al*., 2001; Mojet *et al*., 2003), and ranged from 20 to 1500 mg/L for caffeine and from 0.1 to 5 mg/L for quinine sulphate dihydrate. Samples were presented in random order. Assessors were asked to determine which samples were bitter compared to water. Those who were able to identify the bitterness of samples down to a minimum concentration of 200 mg/L for caffeine and 2 mg/L for quinine sulphate dihydrate were selected. In this way, 14 assessors were selected from 24 participants, based on their sensitivity to the 2 bitter compounds. The selected panel consisted of 8 female and 6 male assessors, of age 19 to 36, who were all students of Wageningen University.

(ii) Training and actual procedure

The 14 selected assessors went through one training session and 2 actual sessions. For these 3 sessions, water and a quinine sulphate dihydrate solution (5 mg/L) were used as standards. Two sets of samples, saponin B and a saponin mixture containing both saponin B and DDMP saponin in the ratio of 1:4 (based on the areas of saponin B and DDMP saponin peaks), were used. Both saponin solutions were tested at 5 different concentrations (2-12 mg/L). All samples were prepared by successive dilution with tap water and were presented in random order. The saponin mixture was extracted from the pea variety *Solara spp* using the above mentioned extraction method and the dried sample was stored at –20 ^{o}C. Saponin B was obtained by boiling the extracted saponin mixture for 8 h, during which DDMP saponin was fully converted to saponin B. In order to verify if the presence of maltol (3-hydroxy-2-methyl-4-pyrone), which remained in the saponin B sample after boiling, would affect the taste of saponins, the selected assessors evaluated maltol (12 mg/L) and saponin B without maltol (2-12 mg/L) for bitterness. Saponin B without maltol was prepared by passing boiled saponin B sample through a *Sep-Pak* C18 solid phase extraction column (Waters Plus tC18 cartridge, 37-55 μm, Etten-Leur, The Netherlands). All sample solutions were prepared just before the start of the sessions. Assessors were presented with samples of 5 mL, which were

swirled in the mouth for 10 sec and then expectorated. The assessors had to score the degree of bitterness for all saponin samples on a 15 cm line scale using water and quinine sulphate dihydrate (5 mg/L) as standards (Line scaling or scoring; Meilgaard *et al*., 1991). The left end anchor of the scale corresponded to no bitterness (water), whereas the right end anchor corresponded to very bitter (5 mg/L quinine sulphate dihydrate solution). Assessors were asked to consume an unflavoured cracker between every single sample in order to remove any persisting bitterness, and to eliminate misleading perception, which might lead to artificial and invalid results. Statistical analysis was performed using SPSS 10.0. The concentrations at which bitterness was perceived were tested by analysis of variance (ANOVA; $\alpha \leq 0.05$).

RESULTS AND DISCUSSION

Bitterness in relation to saponin content

A number of studies have reported on the undesirable bitter taste of saponins, but only a few have actually investigated the sensory characteristics of purified saponins (Price & Fenwick, 1984; Price *et al*., 1985). These two studies both showed that saponins are bitter but it is not known how saponins differ in bitterness. Therefore, the bitterness of the saponin mixture (80% DDMP saponin and 20% saponin B) and saponin B with and without maltol, was evaluated by 14 selected and trained assessors. **Figure 2** (solid line) shows a representative RP-HPLC chromatogram of an extract of one of the pea varieties (*Solara*), which contain saponin B and DDMP saponin, as well as the internal standard (equilenin). Saponin B used for sensory evaluation was obtained by boiling the saponin mixture for 8 h, during which DDMP saponin was quantitatively converted to saponin B (**Figure 2**; dotted line) and maltol.

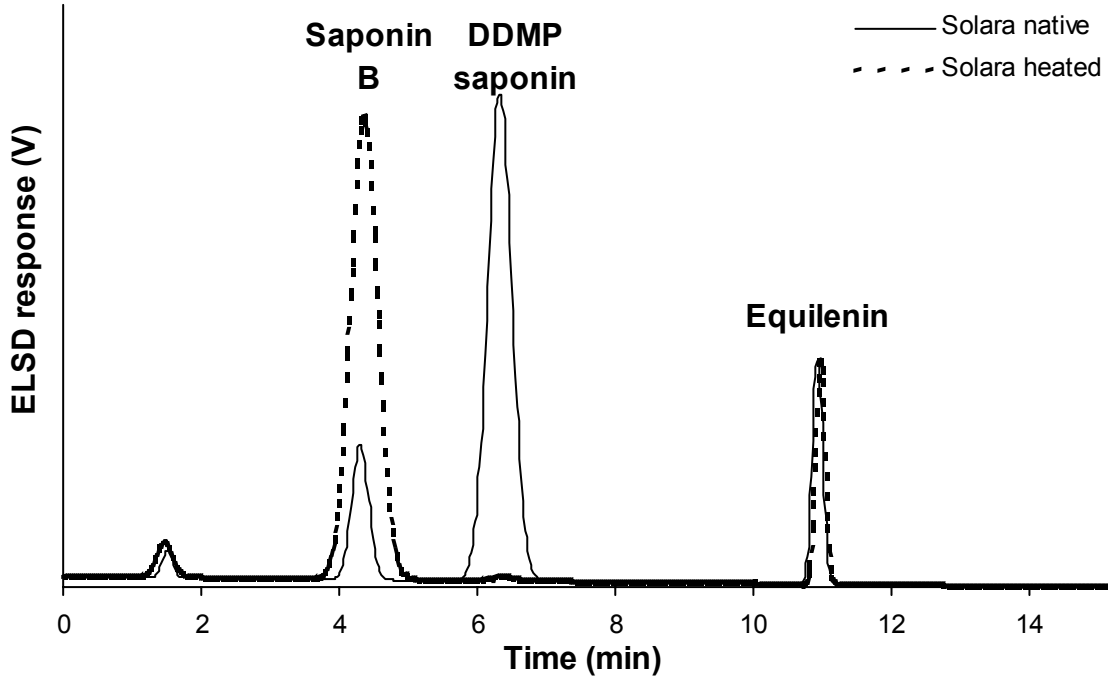


Figure 2: Representative RP-HPLC profiles of saponin B, DDMP saponin and the internal standard, equilenin.

From the evaluation, it was observed that one of the assessors was insensitive to the bitterness of all saponin samples tested and the values from this assessor were, therefore, omitted. **Table 2** and **Figure 3** show the results obtained from the remaining 13 assessors. These results reveal that saponins are indeed bitter to most people in the panel. Samples of saponin B without maltol showed the same bitterness intensity as that of saponin B with maltol, indicating that the released maltol does not affect bitterness. Also, maltol was observed not to taste bitter at a concentration of 12 mg/L (results not shown). The bitterness scores given by the assessors increased with increasing concentration for all saponin samples. This shows that the perceived bitterness is related to saponin concentration within the concentration range studied. Furthermore, most of the assessors (11 out of 13) tasted the bitterness in the extracted saponin mixture solutions at concentrations as low as 2 mg/L, whereas less than half of the assessors (5 out of 13) detected the bitterness of saponin B, with and without maltol, at the same concentration (**Table 2**). Statistical analysis showed that the saponin mixture, and hence, DDMP saponin, was significantly more bitter than saponin B ($\alpha \leq 0.05$) at all concentrations.

Table 2: No of assessors detecting bitterness in saponin solutions (2-12 mg/L)

	No of assessors out of 13 detecting bitterness		
Concentration (mg/L)	**Saponin Mixture**	**Saponin B + maltol**	**Saponin B without maltol**
2	11	5	5
4	12	8	NP
8	13	9	8
10	13	10	11
12	13	11	12

NP: not performed

Aqueous solutions of saponin B, extracted from pea flour at concentrations above 250 mg/L, were found to have both a bitter and an astringent taste (Price & Fenwick, 1984). This concentration is a factor 100 higher than the concentrations used in our study, in which bitterness was already perceived at a concentration of 2 mg/L. Price *et al*. (1985) found that untreated pea flour had a stronger bitterness rating than “debittered” pea flour, which contained half the amount of saponin. These reports demonstrate that bitterness is indeed related to saponin content, as is confirmed by our own sensory tests. In addition, the degree of bitterness was also shown to depend on the quantity and types of saponin present.

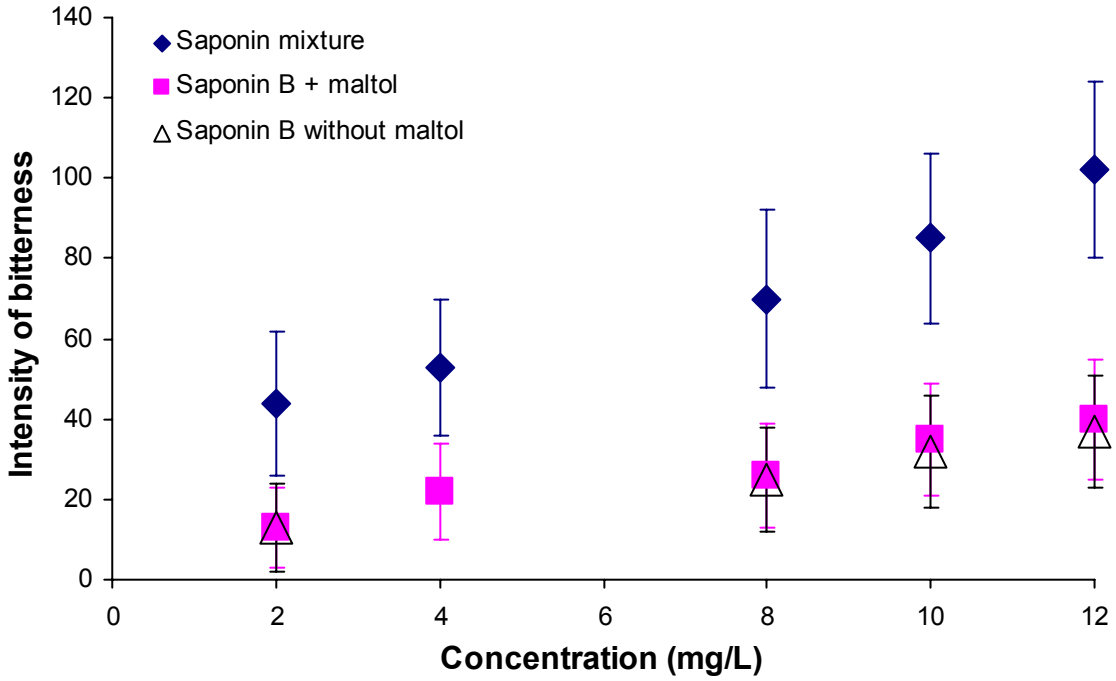


Figure 3: Bitterness intensity of saponins. Intensity of bitterness was obtained by calculating the average of the scores given on a 150 mm line scale for each concentration by the 13 assessors. The error bars represent standard errors.

Threshold levels of DDMP saponin and saponin B

The two bitter compounds used in this study, caffeine and quinine sulphate, are often used as standards in sensory evaluation of bitterness (Mojet *et al*., 2001; Mojet *et al*., 2003). The threshold value (the minimum detectable concentration) of both compounds is remarkably different. There is a factor of 100 difference between the normal practical range of 198-498 mg/L for caffeine and the range of 2-4 mg/L for quinine sulphate (Mojet *et al*., 2003). The range of concentrations used for both saponins in our sensory evaluation was 2-12 mg/L, and was chosen based on the results of preliminary sensory sessions. Bitterness of saponins was already perceived by the sensory assessors at very low concentrations (*e.g*. 2 mg/L), which is similar to the threshold level of quinine sulphate. With the results obtained in **Table 2**, the estimated threshold values for the saponin mixture and for saponin B are < 2mg/L and ~ 8 mg/L, respectively. Knowing the difference in bitterness of saponin B and DDMP saponin, it was of interest to investigate the saponin content and composition of

various pea varieties that have very different characteristics. Therefore, 16 pea varieties were selected (**Table 1**).

Saponin content and composition of pea seeds

Figure 4 shows the amounts of DDMP saponin and saponin B present in 16 pea varieties. It can be seen that DDMP saponin was the predominating saponin in all 16 pea varieties. In 2 varieties (*KPMR 146 India* and *Pisum elatius 1140175)*, DDMP saponin was the only saponin present. The DDMP saponin contents varied from 0.7-1.5 g/kg dry matter (DM), whereas those of saponin B varied from 0-0.4 g/kg DM. The observed variation in the total saponin content among the 16 pea varieties was a factor of almost 3 between the lowest and the highest value. In general, the yellow pea varieties showed lower saponin contents than the green and brown varieties.

It is known that pea varieties may differ in saponin content (Kitagawa *et al*., 1984; Bishnoi & Khetarpaul, 1994; Daveby *et al*., 1997), but the saponin contents reported in literature vary greatly and often depend on the methods used for extraction and quantitative analysis. Saponin contents in the range of 1.1-2.5 g/kg have been found in field and vegetable pea cultivars (Bishnoi & Khetarpaul, 1994), whereas the saponin B contents in 40 Swedish pea varieties were reported to be in the range of 0.8-2.5 g/kg (Daveby *et al*., 1997). In the present study, lower saponin contents were found; 0.7-1.9 g/kg. The lower saponin contents could be due to the different method used for extraction, but it could also be that the tested lines are the result of progressive selection for low bitterness over the years. Saponin B was the only saponin reported to be present in peas in most of the previous studies, except in a study by Daveby *et al*., (1998). In this study, DDMP saponin was also detected, using a relatively mild extraction method. The ratio of saponin B to DDMP saponin found by Daveby *et al*. (1998) was 0.24, after extraction for 30 min. This ratio, however, rose to 0.55 when an extraction time of 24 h was used. Our results confirm that the investigated pea varieties contain predominantly DDMP saponin and only small amounts of saponin B (**Figure 4**), when a mild extraction method was employed. The ratios of saponin B to DDMP saponin ranged from 0.2 to 0.6 with an average of 0.3, in the 16 varieties investigated.

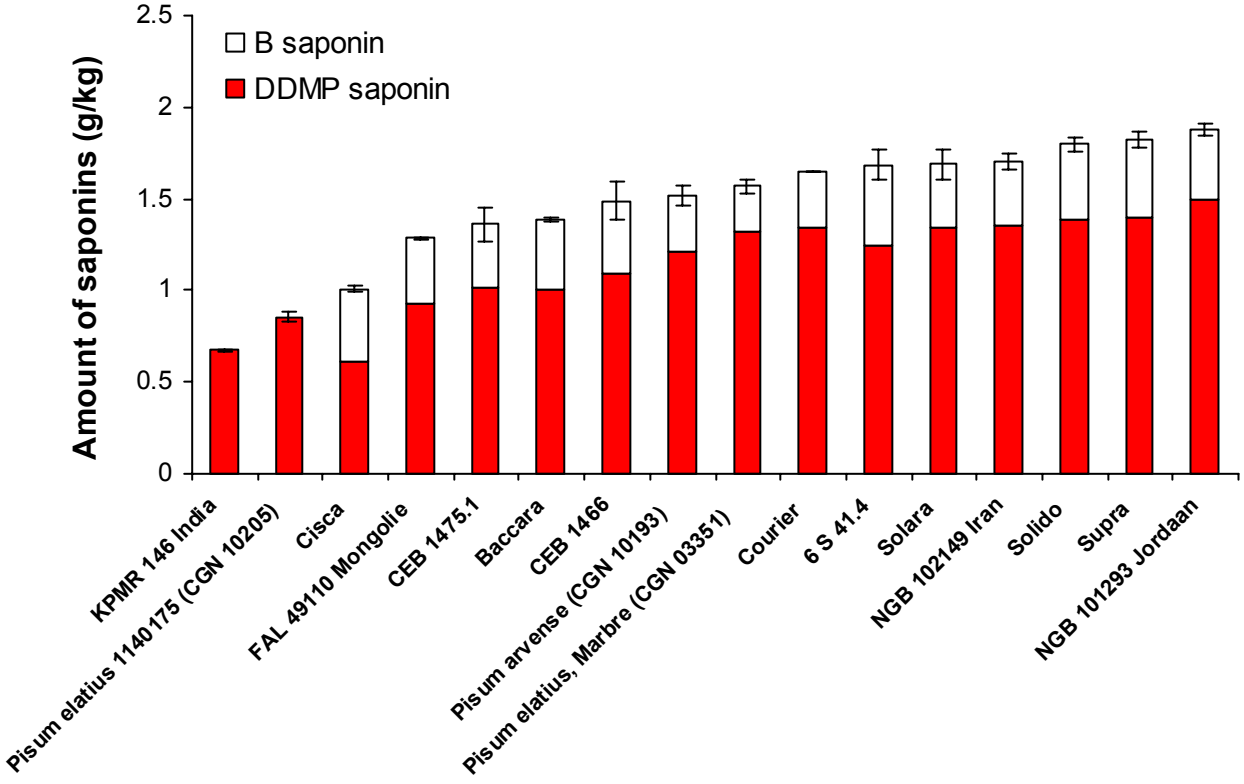


Figure 4: Saponin content and composition of 16 pea varieties.

It can be concluded that peas contain mainly DDMP saponin and only a small amount of saponin B. Both saponins have a bitter taste, of which the intensity increases with concentration, but the DDMP saponin is significantly more bitter than the saponin B. Therefore, pea varieties that have a high DDMP saponin content or a high total saponin content will likely taste more bitter than those low in saponins. Processing of peas and/or its products (*e.g.* boiling) may lead to a decrease in bitterness as DDMP saponin is converted to the less bitter saponin B. As saponins are known to bind to proteins (Potter *et al*., 1993; Ikedo *et al*., 1996; Shimoyamada *et al*., 1998, 2000; Morton & Murray, 2001; Liu *et al*., 2003), it can be expected that protein products such as isolates are enriched in saponins, which may hamper the application of these protein isolates in food products.

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Chapter 4

Saponins stripped down to skeletons

ABSTRACT

Saponins are a structurally diverse class of triterpenoid glycosides, occurring in many plant species. The structures of saponins are reviewed and classified based on four premises: (i) Cyclization patterns of oxidosqualene form the basis of skeletal classification; (ii) Decoration of the skeletons (functional groups, unsaturation, bridging, glycosylation) are excluded, unless they affect the number of rings or ring configuration; (iii) Numbering of carbon atoms in the saponin skeleton is reminiscent of their biosynthetic origin, the backbone of the 22 contiguous carbon atoms of oxidosqualene; (iv) Processing of skeletons (*e.g.* ring expansion, removal of methyl groups reminiscent of oxidosqualene) and their mutual relationships are accounted for. A total of 21 skeletons were documented, which could be classified into 5 main classes: 66666, 66665A, 66665B, 6665A, and 6665B, where the numbers indicate the number of carbons in the subsequent rings. The classes of 6665A and 6665B are further divided into groups and subgroups, because these skeletons are subjected to extensive processing. A relationship between the type of skeleton and the plant origin was drawn. Oleanane was the most common skeleton and is present in most orders of the plant kingdom. Up to 4 main classes of skeleton can exist within one plant order, but the distribution of skeletons in the plant kingdom does not seem to be order-specific. Several substituents (*e.g.* –OH, =O, glycosyl residues, *etc.*) are simultaneously attached to oleanane. Sugar chains of 1 to 8 glycosyl residues can be attached to oleanane, most commonly at C2 and/or C18 of the skeleton. The kind of substituents, as well as the carbon atoms of the skeleton to which they are attached, does not seem to be plant order-specific.

KEYWORDS: *Saponin(s), class(es), squalene, oxidosqualene, triterpenoid, structure(s), skeleton(s), substituent(s), sugar chain(s), unsaturation, phylogenetic tree*

INTRODUCTION

Saponins are generally known as non-volatile, surface-active compounds that are widely distributed in nature, occurring primarily in the plant kingdom (Lasztity *et al*., 1998; Oleszek, 2002). The name 'saponin' comes from the Latin word, *sapo*, which means 'soap', because saponin molecules form soap-like foams when shaken with water. They are structurally diverse molecules that are chemically referred to as triterpene glycosides, which consist of non-polar aglycones coupled with one or more sugar chains (Oleszek, 2002). They have a diverse range of properties that include sweetness and bitterness (Grenby, 1991; Kitagawa, 2002; Heng *et al*., 2005b), foaming and emulsifying properties (Price *et al*., 1987), pharmacological and medicinal properties (Attele *et al*., 1999), as well as insecticidal and molluscicidal activities (Sparg *et al*., 2004). Saponins have found wide applications in beverages and confectionery, as well as in cosmetics (Price *et al*., 1987; Petit *et al*., 1995; Uematsu *et al*., 2000) and pharmaceutical products (Sparg *et al*., 2004).

Several reviews have been published over the last two decades that focused on the biosynthesis, structural elucidation, isolation of saponins, as well as their biological activities (Mahato *et al*., 1988; 1992a; Mahato & Nandy, 1991; Mahato & Sen, 1997; Tan *et al*., 1999; Connolly & Hill, 2000; Sparg *et al*., 2004). These reviews give a dense overview of saponins with all their structural details, yet provide little information on skeletal classification. Saponins are often subdivided into 2 classes, the triterpenoid and the steroidal saponins (Abe *et al*., 1993; Sparg *et al*., 2004). This differentiation is misleading, because steroidal saponins are also derived from the 30-carbon precursor, oxidosqualene (Haralampidis *et al*., 2002). The main difference between the two classes lies in the fact that the steroidal saponins usually have three methyl groups removed (*i.e.* they are mostly C27 molecules), whereas the triterpenoid saponins have not, and remain as C30 molecules. In one recent review (Sparg *et al*., 2004), saponins were classified into 3 classes, namely, the triterpenoid saponins, the spirostanol saponins and the furostanol saponins. This classification, however, does not reflect the diversity of saponins. A class of compounds, which is closely associated with saponins, are the steroidal glycoalkaloids (Haralampidis *et al*., 2002). Although these compounds have the same ancestor as saponins, they will not be considered in this review, because they contain a nitrogen as an intrinsic part of their skeleton. The aim of this review is to classify saponins derived from their 30-carbon precursors that have been found in plants so far, and to give their basic skeletal structures and relationships. Attempts were also made to link the various skeletons to plant orders.

Cyclization of the saponin skeletons from squalene

Saponin biosynthesis proceeds via the isoprenoid pathway in which 3 isoprene units (C5) are linked to each other resulting in a C15 structure, which is subsequently dimerised to give a product of 30 carbon atoms, squalene (Abe *et al*., 1993; Haralampidis *et al*., 2002). Squalene can be oxidized to oxidosqualene, which is the most common starting point for cyclization reactions (**Figure 1**) (Abe *et al*., 1993; Haralampidis *et al*., 2002). Note that the 22 contiguous carbon atoms of oxidosqualene are numbered, and that the carbons of the methyl groups at C1, C5, C9, C14, C18 and C22 of oxidosqualene are not. Oxidosqualene is converted to cyclic derivatives via protonation and epoxide ring opening. The cyclization and subsequent rearrangements can proceed in different ways, and eventually a carbocation needs to be neutralized. Neutralisation usually requires a series of hydride shifts and/or methyl migrations (not indicated in the figure), leading to either the formation of a double bond (elimination reaction), or the formation of a cyclopropanyl ring. The resulting compounds are called triterpenoids. **Figure 1** only shows the cyclization reactions applying to saponin structures that we have found in our literature search[1].

The type of cyclase that is involved in the cyclization reaction primarily determines the skeleton that is formed (**Figure 1**). Many different kinds of cylases (*e.g*. cycloartenol synthase, lanosterol synthase, β-amyrin synthase) have been described, and their mechanism of action is rather well documented (Abe *et al*., 1993; Wendt *et al*., 2000; Haralampidis *et al*., 2002). Cyclization of oxidosqualene can proceed in 2 directions, either via the 'chair-chair-chair' or chair-boat-chair' conformation, which yield different skeletons. An important difference between the two resulting configurations lies in the stereochemistry of the methyl groups at C9 and C14. In the 'chair-chair-chair' conformation, the methyl group at C9 is an up stereo and that at C14 is a down stereo, whereas the opposite is true for the 'chair-boat-chair' conformation. It should be noted that the numbering of the carbon atoms in this scheme is done according to the backbone of oxidosqualene and that the methyl groups are not numbered, which is different from that in the IUPAC[2] nomenclature.

[1] Aglycone structures of saponins were obtained from year 1987 till present from www.sciencedirect.com, using "saponins" and "structures" as key words.
[2] International union of pure and applied chemistry home page; http://www.chem.qmul.ac.uk/iupac/

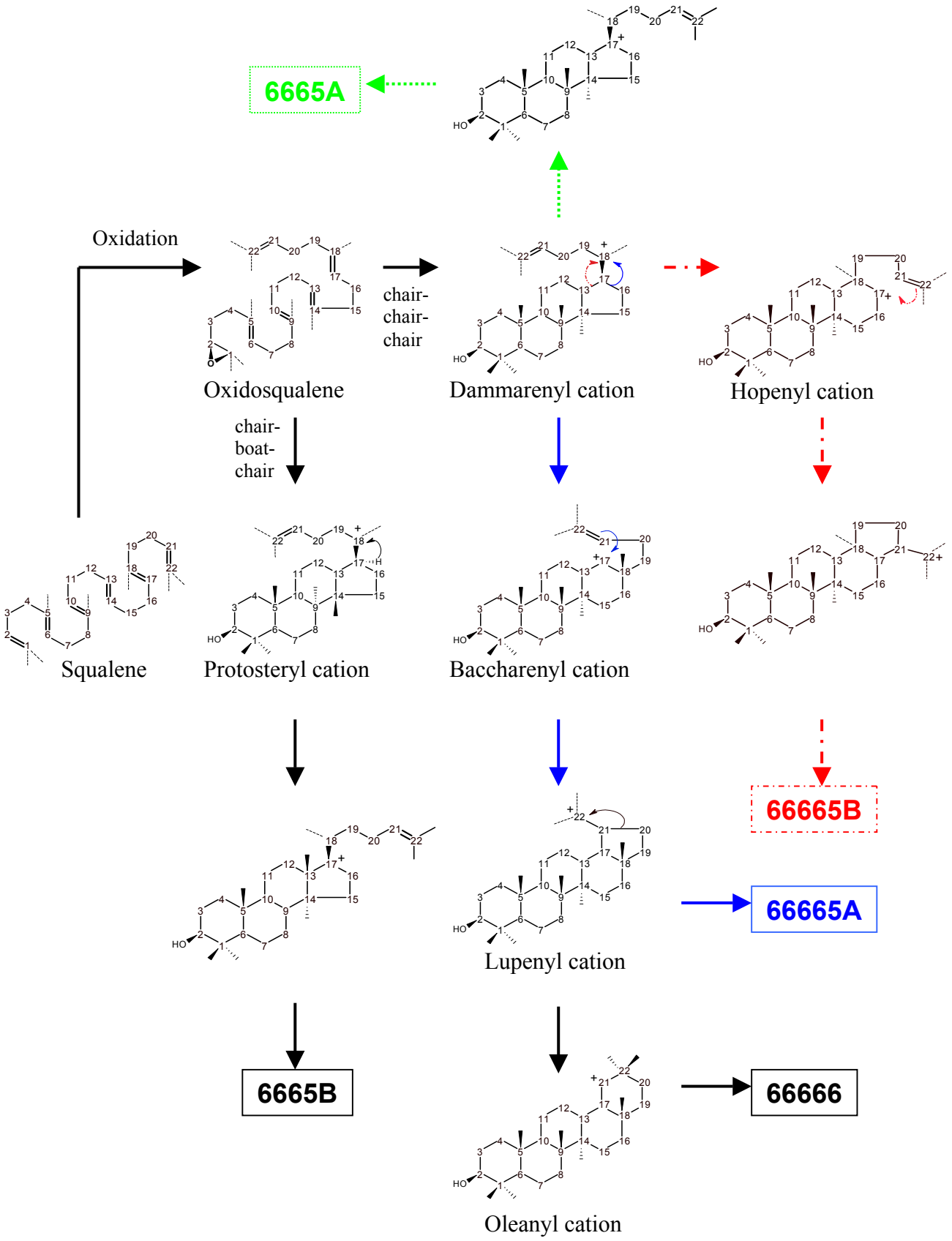


Figure 1: The cyclization of oxidosqualene to various saponin skeletons.

A proton-initiated cyclization via the 'chair-chair-chair' conformation produces the tetracyclic dammarenyl C18 cation, the 5-membered ring of which can expand by a C16-C17 or a C13-C17 attack of the carbocation. The former yields the tetracyclic baccharenyl C17 cation, in which C18 is inserted between C16 and C17 to form a 6-carbon ring. This is followed by rearrangement of the C21-C22 double bond, which produces the pentacyclic lupenyl C22 cation. This lupenyl cation can rearrange further to yield either a 66666 or a 66665A skeleton. This number representation is adopted from the description given by Xu *et al*. (2004), which indicates the number of carbons in the subsequent rings of the skeleton. For example, 66665 indicates that the skeleton is composed of four consecutive 6-carbon rings followed by a 5-carbon ring. The C13-C17-based rearrangement of the dammarenyl cation produces the tetracyclic hopenyl C17 cation, in which C18 is inserted between C13 and C17 to form a 6-carbon ring. The hopenyl cation can subsequently rearrange into a 66665B skeleton. Alternatively, the dammarenyl cation can also rearrange to the tetracyclic 6665A skeleton.

With the 'chair-boat-chair' conformation, a tetracyclic protosteryl C18 cation is formed by the proton-initiated cyclization, ultimately leading to the skeletons 6665B. After cyclization, a series of additional modifications of the triterpenoid follows, eventually yielding saponins. These modifications include various substitutions (with *e.g*. –OH, glycosyl residues), elimination, and/or oxidation reactions. Very little is known about the enzymes and the biological pathways mediating these modifications (Haralampidis *et al*., 2002).

The diversity of saponin aglycones can be seen from several reviews (Agarwal & Rastogi, 1974; Mahato *et al*., 1988; Mahato & Nandy, 1991). Besides the number of rings, the many aglycones identified differ in their number and/or type of substituents attached (*e.g*. –OH, =O, $–CH_3$, $–CH_2OH$, –CHO, –COOH, and or glycosyl residues), the number and position of double bonds, bridging and ring opening, as well as in their stereochemistry. This leads to an extensive collection of aglycones, many of which can have the same basic skeleton. The postulated classification of 3 groups of saponins (Sparg *et al*., 2004) does not accommodate all the aglycone structures reported so far, as there are more than 3 different skeletons known. Moreover, the numbering of carbon atoms of the aglycones according to IUPAC does not reflect their biosynthetic origin, which obscures that certain skeletons have actually similar ancestors, in particular where ring expansion/formation through oxygen insertion has occurred. Therefore, this review seeks to accommodate the existing aglycones in simplified skeletons, according to the following premises:

1) The different cyclization patterns of oxidosqualene form the basis of the skeletal classification.
2) Decoration of the skeletons (functional groups, unsaturation, bridging, glycosylation) is excluded, unless they affect the number of rings or ring configuration.
3) The numbering of carbon atoms in the saponin skeleton is reminiscent of their biosynthetic origin, the backbone of the 22 contiguous carbon atoms of oxidosqualene.
4) Processing of skeletons (*e.g.* ring expansion, removal of methyl groups reminiscent of oxidosqualene) and their mutual relationships are accounted for.

Saponin complexity to skeletal simplicity

In order to illustrate the different carbon numbering employed in this review, two examples are shown in **Figure 2** (Guo & Kenne, 2000; Kennelly *et al*., 1995). The aglycones **2A(i)** and **2B(i)** (both numbered according to the IUPAC nomenclature) were simplified to **2A(ii)** and **2B(ii)** by omitting the oxidosqualene-derived hydroxyl group and the substituents added after cyclization (including double bonds), and re-numbered in **2A(iii)** and **2B(iii)**, respectively, according to their biosynthetic origin, the carbon numbering of oxidosqualene. More specifically, C3-OH, double bond C12-C13, C16-R2, C20-R4 and C22-R3 (where R1-R4 are substituents) have been omitted. R1 always contains a carbon and can be CH_3, CH_2OH or CHO, which are of different degree of oxidation. Methyl groups attached to C1, C5, C9, C14, C18, and C22 are shown, because they occurred in all saponins with this skeleton. Double bonds are not indicated in the skeletal structures, because they can occur at different positions in the molecules, depending on the plant species they originate from. Similarly, in structure **2B(i)**, the C3-COOH, C12-OR (where R is a glycosyl residue), C20-OH, C25-OH, and double bonds C23-C24 and C4-C28 have been omitted to give **2B(ii)**. By employing the above procedure, a saponin structure is reduced to a skeleton.

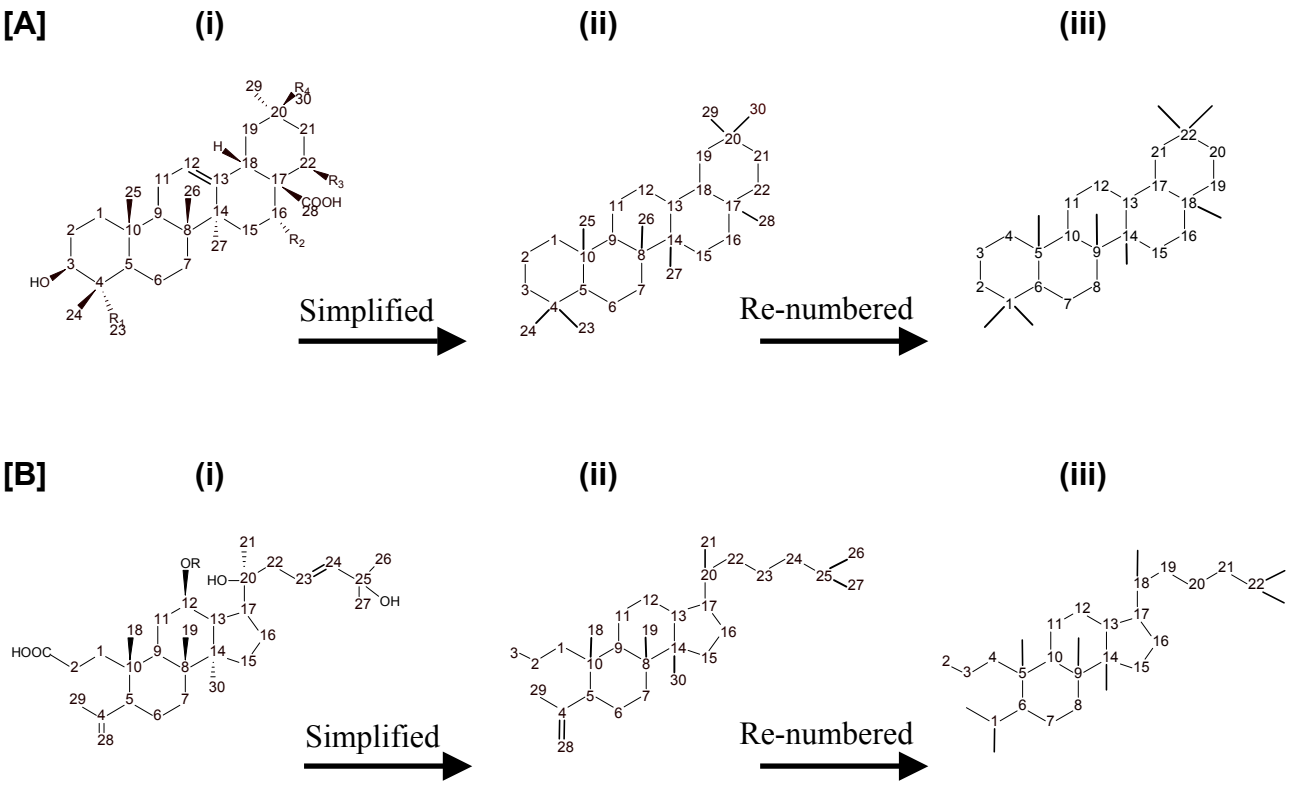
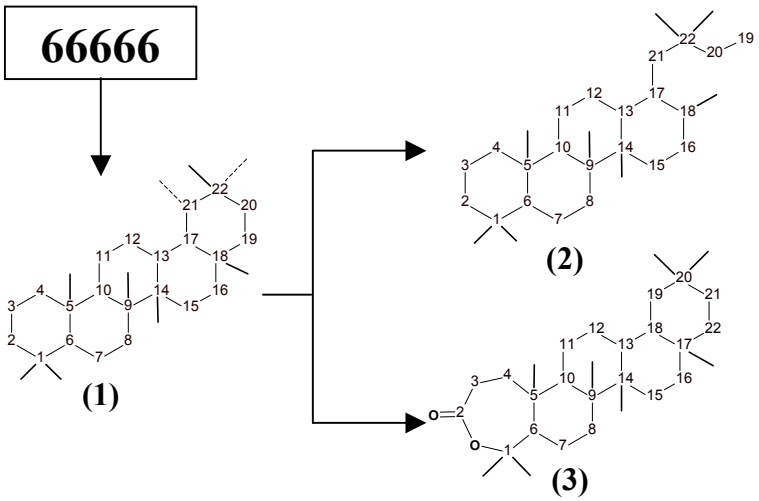


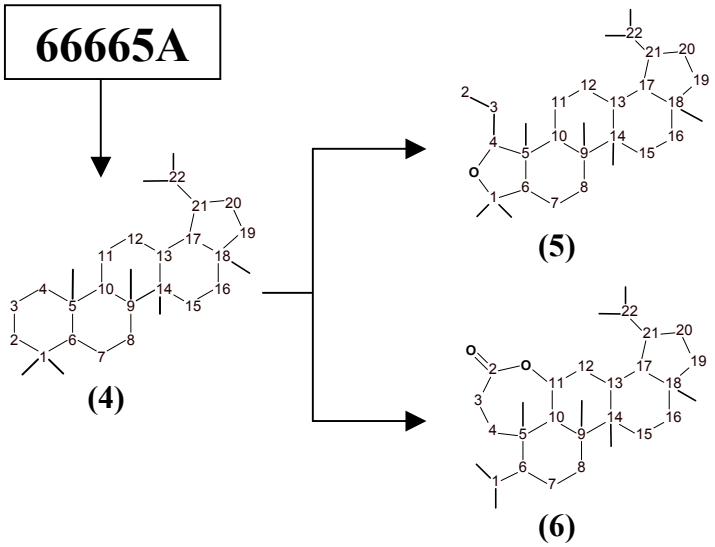
Figure 2: Simplifying and numbering of carbon atoms for the [A] oleanane skeleton and [B] secodammarane skeleton. R1, R2, R3 and R4 in A(i) represent different substituents. R1 can be CH_3, CH_2OH or CHO. R2 and R3 can be H or OH. R4 can be CH_3 or $COOCH_3$. R in B(i) represents a glycosyl residue and can be quinovose or arabinose.

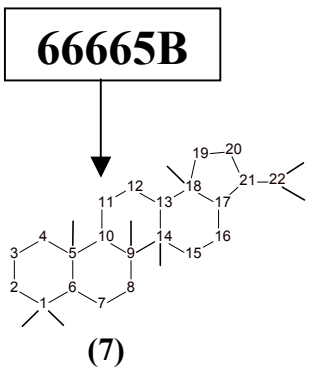
Six skeletons form the basis for saponin structure

The 21 skeletons shown in **Figure 3** were classified into five main classes, 66666, 66665A, 66665B 6665A and 6665B. Each class contains skeletons that either have similar number representation or can be derived from each other. The various classes can be divided in groups and subgroups, particularly 6665A and 6665B, which seem to be subjected to extensive processing. The numbering of the carbon atoms of the skeletons is according to their biosynthetic origin (**Figure 1**). Saponins containing skeletons from classes 66666, 66665A, 66665B and 6665A, as well as skeletons (20) and (21), are generally considered as triterpenoid saponins, whereas those from class 6665B are known as steroidal saponins.

Figure 3







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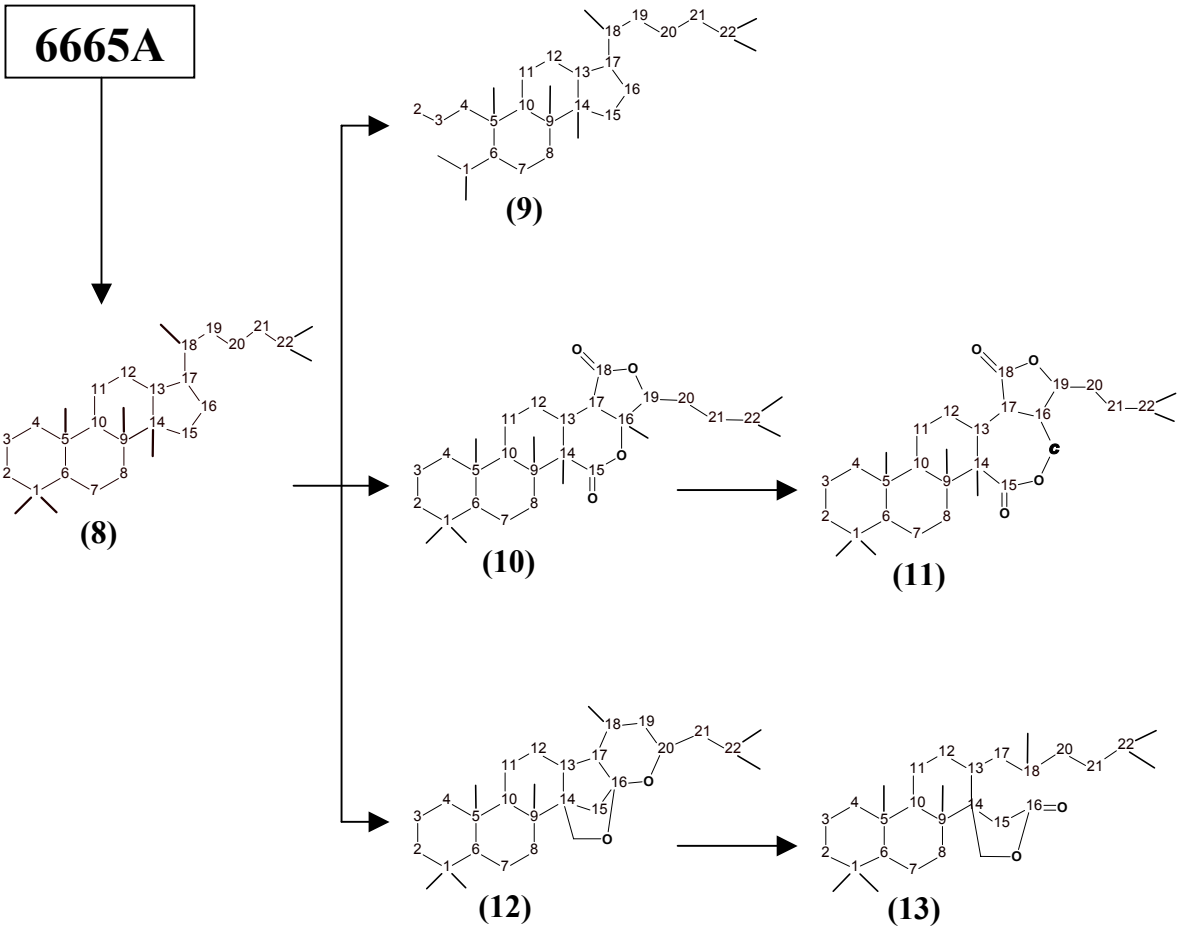

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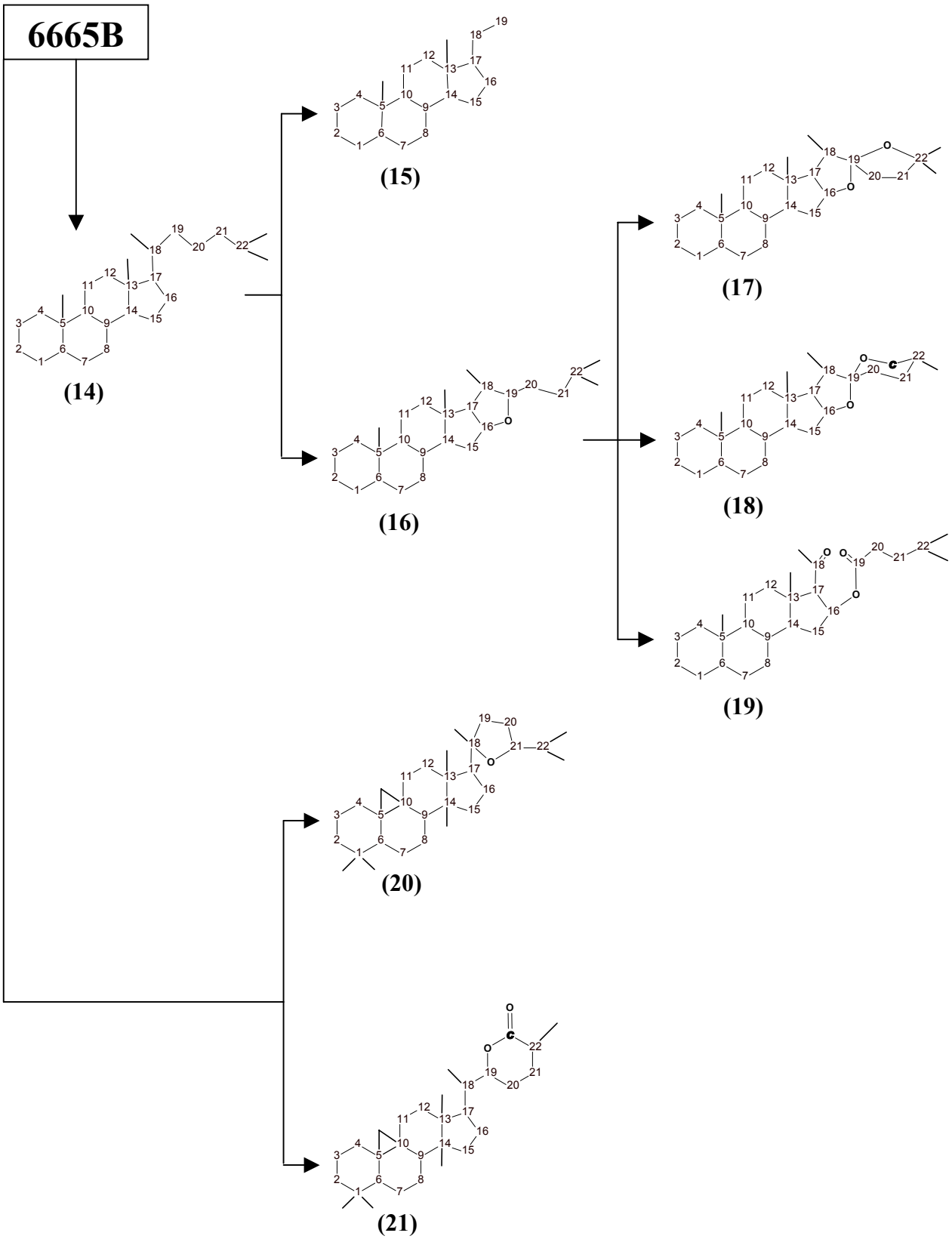


Figure 3: Structures of 21 skeletons classified into 5 main classes, 66666, 66665A, 66665B, 6665A, and 6665B, and their biosynthetic relationships. Note that the carbon numbering reflects their original position in the oxidosqualene precursor, which is different from the normally adopted IUPAC numbering. Trivial names are given to the skeletons: (1) Oleanane; (2) Steganogenin; (3) A-homo-3a-oxa-5β-olean-12-en-3-one-28-oic acid; (4) Lupane; (5) Isochiisanogenin; (6) Chiisanogenin; (7) Hopane; (8) Ginsenoside; (9) Secodammarane; (10) & (11) Dammarane; (12) Jujubogenin; (13) Trans ebelin-lactone; (14) Cholestane; (15) Pregnane; (16) Furostane; (17) Furospirostane; (18) Spirostane; (19) 16-B-hydroxypregnenolone; (20) & (21) Cycloartane. The ***C*** in skeletons (11), (18) and (21) represents a carbon (usually a methyl group originally present in oxidosqualene), which has become part of a ring during processing of these skeletons.

(i) Class 66666

Skeletons (1), (2) and (3) have the basic 66666 skeleton. Skeleton (1) is the most common skeleton found in saponins (Xu *et al*., 2004). Saponins containing this skeleton are generally known as oleanane type saponins (Sparg *et al*., 2004) and have been isolated from a wide array of plants (Daveby *et al*., 1998; Woldemichael & Wink, 2002; Treyvaud *et al*., 2000; Voutquenne *et al*., 2003; Wandji *et al*., 2003). Most of the skeleton (1) structures have two methyl groups at C22, *i.e*. the β-amyrin skeleton, and occasionally, one of these two methyl groups can be processed (Ikuta *et al*., 1991; Miyakoshi *et al*., 1997; Park *et al*., 2002); for instance, a hydroxyl group can occupy the position of the methyl group (Park *et al*., 2002). In some cases, one of the two methyl groups migrates to C21 as part of the mechanism neutralizing the carbocation, giving the α-amyrin skeleton (Amimoto *et al*., 1993; Zhao *et al*., 1997; Sahpaz *et al*., 2000). Therefore, the C21 and one of the C22 methyl groups are indicated with dotted lines in Figure 3. The α-amyrin and β-amyrin skeletons are the cyclization products of distinct cyclases, α-amyrin synthase and β-amyrin synthase, respectively (Haralampidis *et al*., 2002). Skeletons (2) and (3) are both derived from skeleton (1). Skeleton (2) has the trivial name, steganogenin, and was thought to be formed by fragmentation of the C18-C19 bond of skeleton (1) (Lavaud *et al*., 1992). Skeleton (3) has an oxygen insertion into its first ring, which was suggested to be a result of enzymatic hydroxylation and oxidation reactions of C1 and C2, respectively (Debella *et al*., 2000; Shirane *et al*., 1996), followed by the formation of an intramolecular ester.

(ii) Class 66665A and 66666B

Skeletons (4), (5), (6) and (7) have the basic 66665 skeleton and have the trivial names, lupane, isochiisanogenin, chiisanogenin and hopane, respectively (Meselhy, 1998; Shirasuna *et al*., 1997; Melek *et al*., 2002). Skeletons (4), (5) and (6) belong to class 66665A, whereas skeleton (7) belongs to a separate class, 66665B, because its last ring is cyclized in a different way (**Figure 1**) (Meselhy, 1998). Both skeletons (5) and (6) are derived from skeleton (4), likely through oxidation/hydroxylation processes (Shirasuna *et al*., 1997), including the oxidation of C2 to a carboxyl group (not shown in skeleton (5)). The result can be an oxygen-containing 4-carbon ring (skeleton (5)) or an oxygen-containing 6-carbon ring (skeleton (6)). Skeletons (3) and (6) have an intramolecular ester (involving the C2 carboxyl group) in common, but the oxygen-containing ring is closed differently, probably depending on the substituent attached to C1 and C11, respectively.

(iii) Class 6665A

Class 6665A contains skeletons (8) to (13), which have the basic 6665 skeleton. Class 6665A is divided into 3 groups, two of which can be further processed. The ginsenoside skeleton (8) (Park *et al*., 1996; Sun & Chen, 1997; Gillis, 1997) is likely to be the precursor of all saponins in this group. It contains an aliphatic chain attached to its 5-carbon ring. The secodammarane skeleton (9) (Kennelly *et al*., 1995; Aoki *et al*., 1988) has a similar structure as skeleton (8), except that its first ring was opened between C1 and C2. The dammarane skeleton (10) contains two ring oxygens, one in the fourth 5-carbon ring and the other in the last 4-carbon ring (Oulad-Ali *et al*., 1994). These heterocyclic rings probably result from oxidation/hydroxylation reactions, similar to those described for skeletons (3) and (6), leading to the formation of two intramolecular esters. Skeleton (10) can undergo ring expansion to give skeleton (11), in which the carbon atom of the methyl group attached to C16 of skeleton (10) becomes a member of the fourth ring (Oulad-Ali *et al*., 1994). The origin of the methyl group at C16 is unclear. It is possible that the methyl group at C18 of skeleton (8) is removed, and that a new methyl group is attached at C16. However, it could also be that this methyl group migrates from C18 to C16 during cyclization by a mechanism still unknown. In the latter case, skeletons (10) and (11) should form a separate group in the 6665A class (or form a class of their own), because they are the products of a different cyclase. Skeleton (12), named jujubogenin, contains a bridge that links C14 to C16 by an oxygen atom (Jain & Kulshreshtha, 1993; Rastogi *et al*., 1994; Mahato *et al*., 2000; Chakravarty *et al*., 2001). This skeleton can be transformed to skeleton (13) by acid-catalysed rearrangement resulting in ring opening (Rastogi *et al*., 1994; Mahato *et al*., 2000).

(iv) Class 6665B

Class 6665B contains skeletons (14) to (21), which also have the basic 6665 skeleton, but differ from those of class 6665A in their biosynthetic origin (**Figure 1**). Class 6665B is divided into 2 main groups, one of which (skeleton (14)) can be subjected to extensive processing. Skeleton (14), known as cholestane, has a similar structure as skeleton (8) in class 6665A, except for the removal of the methyl groups at C1 and C9, and the migration of the C13 methyl group from C14 (Huang *et al*., 2000; Pires *et al*., 2002). Skeleton (15) is derived from skeleton (14) by reduction of the length of the aliphatic chain (Dong *et al*., 2001). Compounds containing skeleton (15) are usually called pregnane glycosides (Achenbach *et al*., 1996; Mimaki *et al*., 1997; Dong *et al*., 2001; Zheng *et al*., 2004), but they have also been referred to as saponins (Uddin Ahmad *et al*., 1998). Skeleton (16) is probably formed from skeleton (14) by hydroxylation or oxidation of C16 and C19, and subsequent ring closure yielding the oxygen-containing 4-carbon ring. The aliphatic C20-C22 chain of skeleton (16) can be processed to give an extra 4-carbon or 5-carbon ring, skeletons (17) and (18), respectively (Mimaki *et al*., 1995; Dong *et al*., 2001). The mechanism by which this is effected is unknown, but probably requires hydroxylation of C19 and C22, or C19 and one of the C22 methyl groups, for skeletons (17) and (18), respectively. Saponins containing skeletons (16) and (18) are termed furostanol and spirostanol saponins, respectively (Sparg *et al*., 2004; Mimaki *et al*., 1999; Wang *et al*., 1997a; Achenbach *et al*., 1994; Onning *et al*., 1993a/b; Onning & Asp, 1993). They have been referred to as different classes by Sparg *et al*. (2004); however, according to our nomenclature, they belong to the same class. Skeleton (19) is processed from skeleton (16) by enzymatic oxidation and ring opening (Dong *et al*., 2001).

Saponins containing skeletons (20) and (21) are named cycloartane saponins. The presence of the cyclopropanyl indicates that these saponins, compared to skeleton (14), are cyclized by different kinds of cyclases (cycloartenol and lanosterol synthase, respectively), which differ in the way that they neutralize the carbocation (formation of cyclopropanyl and elimination, respectively). In skeletons (20) and (21), the aliphatic C17-C22 is processed differently after cyclization of the skeleton. Skeleton (20) has an oxygen-containing 4-carbon ring linked to C17 of the skeleton (Verotta *et al*., 1998; 2001), which is probably formed in a similar way as that of skeleton (16). Skeleton (21) is similar to skeleton (20), except that it has an oxygen-containing 5-carbon ring linked to C17 through a methylene spacer (Choi *et al*., 1989; Kennelly *et al*., 1996). Note that one of the C22 methyl groups participates in ring formation. Ring closure is achieved through the

formation of an intramolecular ester, probably following similar mechanisms as described for skeletons (3), (6), and (10).

Distribution of saponin skeletons in the plant kingdom

With the isolated aglycones summarised into basic skeletons in **Figure 3**, it is of interest to know the relationship between the plant origin (taxonomy) and the type of skeleton. Plants can be classified based on their physical characteristics and are hierarchically divided into kingdom, sub-kingdom, division, class, subclass, order, family, genus and species. **Figure 4** shows the phylogenetic tree of the various plants from which saponins have been isolated. As the numbers of plant species that have been isolated so far are numerous, the phylogenetic tree presented in **Figure 4** covers only from the kingdom to the order.

From **Figure 4**, it can be seen that saponins are present in 2 major classes within the plant kingdom, the Magnoliopsida and the Liliopsida. Magnoliopsida is the class of dicotyledons (*i.e*. plants with 2 leaves formed on a seedling), whereas Liliopsida is the class of monocotyledons (*i.e*. 1 leaf). Both classes belong to the sub-kingdom, Tracheobionta, which are all vascular plants, and the division, Magnoliophyta, which are the flowering plants or the angiosperms. Within the class of Magnoliopsida, there are 6 sub-classes, namely, Asteridae, Caryophyllidae, Dilleniidae, Hamamelidae, Magnoliidae and Rosidae. Within the class of Liliopsida, there are 3 sub-classes, namely, the Commelinidae, Liliidae and Zingiberidae. Each sub-class contains 1 or more orders. Asteridae and Rosidae are the most abundant sub-classes consisting of 7 and 9 orders, respectively.

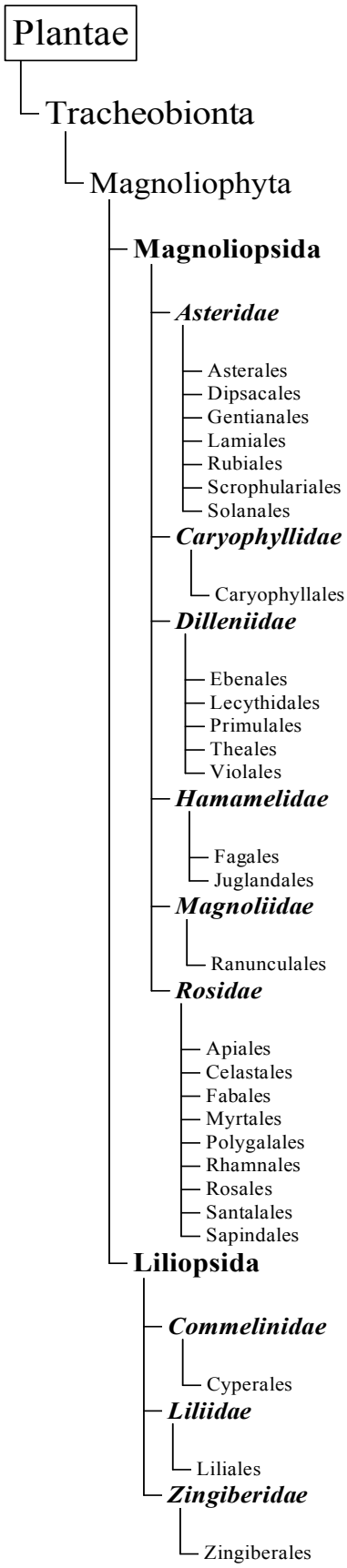


Figure 4: Phylogenetic tree showing the plant orders from which saponins have been isolated and characterised.

Table 1: Distribution of the various skeletons in the plant kingdom

Plant subclasses	Plant orders	No. species	Skeletons				
			66666	66665A	66665B	6665A	6665B
Class of Magnoliopsida							
Asteridae	Asterales	**15**	1				
	Dipsacales	**6**	1	4			
	Gentianales	**1**	1				
	Lamiales	**4**	1				
	Rubiales	**10**	1			8	15
	Scrophulariales	**5**	1			12,13	
	Solanales	**2**					16,18
Caryophyllidae	Caryophyllales	**24**	1	4	7		
Dilleniidae	Ebenales	**8**	1				
	Lecythidales	**1**	1				
	Primulales	**8**	1				14
	Theales	**1**	1				
	Violales	**8**	1			8	
Hamamelidae	Fagales	**1**				9	
	Juglandales	**1**				9	
Magnoliidae	Ranunculales	**10**	1				
Rosidae	Apiales	**22**	1, 2	4,5,6		8	
	Celastales	**7**	1				
	Fabales	**31**	1, 3	4		8	14,16,18,20,21
	Myrtales	**1**	1				
	Polygalales	**1**	1				
	Rhamnales	**3**				10,11,12	16
	Rosales	**3**	1				
	Santalales	**1**	1				
	Sapindales	**18**	1				14,15,16,18
Class of Liliopsida							
Commelinidae	Cyperales	**2**	1				14,18
Liliidae	Liliales	**41**	1			8	14,15,16,17,18,19
Zingiberidae	Zingiberales	**2**					16

The distribution of the various skeletons in different plant orders is presented in **Table 1**. The number of plant species of which saponins have been isolated is indicated for each order. Liliales is the most abundant one comprising of 41 different species, followed by the Fabales, having 31 species. It can be seen that skeleton (1) is the most common skeleton occurring in almost all orders, except Solanales, Fagales, Juglandales, Rhamnales and Zingiberales. The absence of skeleton (1) in these 5 orders may suggest that it has not been analysed for, and does not necessarily mean that it is not present. In half of the 25 orders in the class of Magnoliopsida, skeleton (1) is the only skeleton present, namely, Asterales, Gentianales, Lamiales, Ebenales, Lecythidales, Theales, Ranunculales, Celastales, Myrtales, Polygalales, Rosales and Santalales.

The sub-classes of Rosidae and Liliidae show the largest diversity in saponin skeletons (**Table 1**), in which the Fabales have skeletons from classes 66666, 66665A, 6665A and 6665B, and the Liliales has skeletons from classes 66666, 6665A and 6665B. The order of Fabales is the most diverse, containing 9 different skeletons, and is followed by Liliales and Apiales, which has 8 and 6 different skeletons, respectively. It can be noticed that processed skeletons always occur with their precursors in one order. For example, the Fabales contain skeleton (3), which is derived from skeleton (1) (Debella *et al*., 2000), and skeleton (18), which is derived from skeleton (16) (Sparg *et al*., 2004). It should be noted that skeletons (20) and (21), which are present in the same plant order, do not coexist at the species level (Pistelli *et al*., 1998; Choi *et al*., 1989; Kennelly *et al*., 1996); they also do not seem to coexist with skeleton (14) derived saponins.

The distribution of saponin skeletons does not seem to be plant order-specific. It should be noted that the saponins were isolated from different parts of a plant, which include the roots, stems, bark, leaves, seeds and fruits. Occasionally, the whole plant was used. We have not observed that specific saponin skeletons are associated with particular parts of a plant; saponins of the same skeleton can be obtained from various plant parts.

Decoration of skeleton (1) saponins

(i) Type of functional groups

As the synthesis of saponins involves the attachment of various functional groups at different carbon positions of the skeletons, a summary of the various substituents and their associated carbon atoms is presented in **Table 2** for the different plant orders. This table only shows the substituents present in saponins having the skeleton (1), the most common skeleton found in the plant kingdom.

Several substituents are attached to the same skeleton. At each carbon atom, more than 1 type of substituent has been reported, which include –OH, =O, –COOH, *etc*.

From **Table 2**, it can be seen that C1, C18, C19, C20 and C22 show the largest diversity in substituents attached. The OH group is distributed throughout all carbon atoms and plant orders. The methyl groups originally present in oxidosqualene often serve as a point of attachment of functional groups. Of these methyl groups, the ones attached at C9 and C21 (only present in the α-amyrin type) is not processed in any of the plant orders, whereas C5 shows only processing in the Fabales (*Periandra dulcis* (Hashimoto *et al*., 1980; 1982; 1983; Suttisri *et al*., 1993)); the C14 methyl groups are moderately processed, whereas those at C1, C18, and C22 are heavily processed. Often these methyl groups are oxidized to different degrees, which are reflected by the presence of CH_2OH, CHO and COOH.

C4 and C12 are found to contain substituents in only 1 plant order. The saponin that contains an OH at C4 was isolated from *Gambeya boukokoensis* (Wandij *et al*., 2003). The other substituent, OH at C12, has been ascribed to *Ilex kudincha* (Ouyang *et al*., 1996). No particular trend between the type of substituents at different carbon positions and the type of plant can be seen, except for the fact that the class of Liliopsida has fewer substituents, which seem to have a lower degree of oxidation than those of the Magnoliopsida.

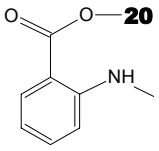
Table 2: Substituents attached to carbon atoms of skeleton (1) in different plant orders

Plant subclasses	Plant orders	Substituents																	
		C1(*4*)[a; b]	**C2(*3*)**	**C3(*2*)**	**C4(*1*)**	**C5(*10*)**	**C7(*6*)**	**C9**	**C11**	**C12**	**C13**	**C14**	**C15**	**C16**	**C18(*17*)**	**C19(*22*)**	**C20(*21*)**	**C21(*19*)**	**C22(*20*)**
Magnoliopsida																			
Asteridae	Asterales	CH_3; CH_2OH; COOH; OH	OH	OH		CH_3		CH_3						=O; OH	CH_3; COOH*; OH		OH	CH_3; OH	CH_3; OH
	Dipsacales	CH_3; CH_2OH; OH	OH			CH_3		CH_3											CH_3
	Gentianales	CH_3; CH_2OH; OH	OH			CH_3		CH_3						OH	CH_2OH; COOH		OH; Benzoyl		CH_3
	Lamiales	CH_3; CH_2OH; OH	OH	OH		CH_3	OH	CH_3	CH_2OH			CH_3		OH	CH_2OH; COOH				CH_3
	Rubiales	CH_3; CH_2OH; COOH; OH	OH	COOH*; OH		CH_3	OH	CH_3				CH_2OH; COOH		OH	COOH*			=O; OH	CH_3; =O; OH
	Scrophulariales	CH_3; CH_2OH; COOH*	OH	OH		CH_3		CH_3	CH_2OH			CH_3			OH		OH	CH_3; OH	CH_3; CH_2OH
Caryophyllidae	Caryophyllales	CH_3; CH_2OH; CHO; OH	CH_2OH; =O; OH	CH_3; OH		CH_3	OH	CH_3			OH	CH_3		OH	CH_3; CH_2OH; COOH*		OH	CH_3; OH	CH_3; COOH*; =O
Dilleniidae	Ebenales	CH_3; CH_2OH; COOH; OH	CH_2OH; =O; OH	OH	OH	CH_3; OH	OH	CH_3						OH	COOH; OH	COOH*; OH; Tigloyl	COOH*; OH; Hexenoyl; Tigloyl	CH_3; OH	CH_3
	Lecythidales	CH_3	OH			CH_3		CH_3						OH	OH	COOH*; OH; Tigloyl	COOH*; OH; Hexenoyl; Tigloyl		CH_3
	Primulales	COOH	OH	OH		CH_3		CH_3			COOH*; OH	CH_3; CH_2OH		COOH*; =O; OH	CH_2OH; CHO; OH	OH; Angeloyl; Cinnamoyl; Tigloyl	COOH*; OH; Angeloyl; Benzoyl		CH_3; CH_2OH; CHO; COOH
	Theales	CH_3; COOH*	OH			CH_3		CH_3					OH	COOH*; OH	CH_2OH	OH; 2-methyl-butanoyl	OH; Angeloyl		CH_3
	Violales	CH_3; OH	OH	OH		CH_3	OH	CH_3											CH_3

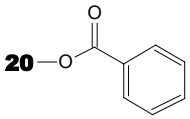
Magnoliidae	Ranunculales	CH_3; CH_2OH; OH	OH		CH_3		CH_3		OH	CH_3		OH	COOH*				CH_3; COOH*; =O; OH
Rosidae	Apiales	CH_3; CH_2OH#; CHO; COOH*; OH	COOH*; OH	CH_3; COOH*; OH	CH_3		CH_3	CH_2OH; =O; OH	OH	CH_3	OH	OH	CH_2OH; COOH* OH	OH	OH	CH_3	CH_3; CH_2OH; COOH*; =O; OH
	Celastales	CH_3; CH_2OH; CHO; COOH*	$COCH_3$; OH		CH_3		CH_3	OH		CH_3					OH	CH_3; OH	CH_3; OH
	Fabales	CH_3; CH_2OH#; COOH; OH	CH_2OH; COOH*; OH	OH	CH_2OH; CHO; COOH*; OH		CH_3	$=CH_2$; =O		CH_3		OH	CH_3; COOH*	DDMP; =O; OH	OH; $=CH_2$		CH_3; CH_2OH; COOH*
	Myrtales	CH_3	OH		CH_3		CH_3										CH_3
	Polygalales	CH_3; COOH	OH	OH	CH_3		CH_3			CH_2OH							CH_3
	Rosales	CH_3; CHO	OH	OH	CH_3		CH_3				OH	OH	COOH; OH	OH	OH	CH_3; OH	CH_3; COOH
	Santalales	CH_3; CH_2OH	OH		CH_3		CH_3										CH_3
	Sapindales	CH_3; CH_2OH; CHO; COOH; OH	OH	COOH*; OH	CH_3		CH_3			CH_2OH; COOH*	OH	COOH*; OH	CH_2OH; COOH*; OH	COOH*; OH; Angeloyl	OH; Angeloyl; Tigloyl	CH_3	CH_3; =O; OH
Liliopsida																	
Commelinidae	Cyperales	CH_3; OH	OH		CH_3		CH_3					OH	CH_3		OH; Benzoyl; N-methyl anthranilic acid		CH_3; CHO
Liliidae	Liliales	CH_3; CH_2OH; OH	OH	OH	CH_3		CH_3					OH	COOH				CH_3

*Indicates that an alcohol can be ester-linked. #Indicates that an acid can be ester-linked. [a]Shaded columns indicate the carbon positions containing the methyl groups that were originally present in oxidosqualene; Note that the C21 methyl group has migrated from C22, as in α-amyrin. [b]Number within bracket indicates IUPAC numbering. Substituents underlined with a dotted line can exist either independently or as part of a bridge, whereas those underlined with a solid line are only encountered as part of a bridge.

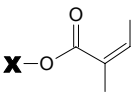
The C19 and C20 can contain various uncommon groups, which are illustrated in **Figure 5**. Substituents at C19 can be liable to chemical reactions *e.g*. DDMP (2,3-dihydro-2,5-dihydroxy-6-methyl-4*H*-pyran-4-one) (Massiot *et al*., 1996; Okubo *et al*., 1996; Heng *et al*., 2005a). DDMP is most commonly found in saponins isolated from legumes such as peas (Daveby *et al*., 1998; Heng *et al*., 2004; 2005a/b), soybeans (Decroos *et al*., 2005; Berhow *et al*., 2002; Hu *et al*., 2002) and lentils (Ruiz *et al*., 1997). Being the native saponin found in soy and peas (Daveby *et al*., 1998; Hu *et al*., 2002; Decroos *et al*., 2005; Heng *et al*., 2004; 2005a/b), DDMP saponin has been the focus of saponin studies as it was found to be unstable at various conditions (Okubo *et al*., 1996; Hu *et al*., 2002; Heng *et al*., 2005a). The ether bond that links the DDMP group to the saponin aglycone at the C19 position, is sensitive to acid and base conditions, as well as prolonged exposure to aqueous environment (Heng *et al*., 2005a). It might be expected that substituents attached by the same or similar bonds (ether or ester linkages) are vulnerable during extraction and/or analytical procedures, which may lead to underestimation of their abundance in plant extracts.



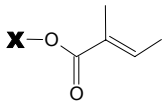
N-methyl anthranilic acid



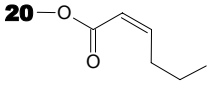
Benzoyl



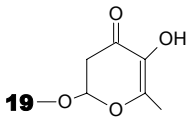
Angeloyl



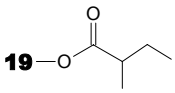
Tigloyl



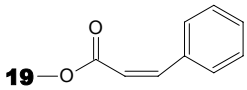
Hexenoyl



DDMP



2-methylbutanoyl



Cinnamoyl

Figure 5: Structures of substituents at C19 and C20 of oleanane skeleton (1). 19 and 20 indicate the position of the oleanane skeleton at which substituent is attached. ***X*** represents both positions 19 and 20 of the oleanane skeleton.

(ii) Bridging

Certain substituents (**Table 2**) attached to skeleton (1) may react with each other, leading to the formation of bridges. The substituents that can be involved in bridge formation are usually those at C13, C18, C20 and C22. The most common bridging is that of C13-O-CH_2-C18, which is formed between a C13-OH and a C18-CH_2OH (Pistelli *et al*., 1996; Ebata *et al*., 1996; Huang *et al*., 2000; Sánchez-Contreras *et al*., 2000). Another example of an ether-linked bridge is C13-O-CH(OH)-C18 (Germonprez *et al*., 2004; Huang *et al*., 2000), which is formed by C13-OH and C18-CHO. Ester-linked bridges also occur, such as C13-O-C(=O)-C18, which is formed by C13-OH and C18-COOH (Ikuta & Morikawa, 1992; Yu *et al*., 1995; Marx Young *et al*., 1997; Gromova *et al*., 1998). It can be seen from **Table 2** that the hydroxyl group at C13 never occurs in a free form, but that it is always engaged in a bridge with C18, either by an ether or an ester link (indicated by the solid underlining). Substituents attached to C18 can also form bridges with other carbon atoms of the oleanane skeleton, such as C18-CH_2-O-CH(OH)-C22 (Lavaud *et al*., 1994), C18-C(=O)-O-C20 (Sakai *et al*., 1999), C18-C(=O)-O-C22 (Ouyang *et al*., 1996; Aquino *et al*., 2001), C18-CH_2-O-C(=O)-C22 (Yayli *et al*., 1998), and C18-C(=O)-CH_2-C20 (Garai & Mahato, 1997). The latter is probably formed by C18-COOH and C20=CH_2. Bridging in the first ring has also been shown to occur, C1-CH_2-O-C5 is formed between C1-CH_2OH and C5-OH (Sahu, 1996). Interestingly, bridging only seems to occur in the class of Magnoliopsida, and not in the class of Liliopsida, which seems to be consistent with the observation that the substituents of the latter have a lower degree of oxidation.

(iii) Unsaturation

Unsaturation (double bonds) is usually a feature that results from the neutralization of a carbocation by an elimination reaction (**Figure 1**). The fact that two double bonds can occur in skeleton (1) saponins, indicates that additional desaturation reactions occur downstream the cyclization process. The single and/or double unsaturation can exist at different positions in the skeleton. Therefore, the double bonds were not indicated in the structures in **Figure 3**. Single unsaturation was found at position C12-C13 (Larhsini *et al*., 2003; Voutquenne *et al*., 2003; Wandji *et al*., 2003; Woldemichael *et al*., 2003), C13-C14 (He *et al*., 1996; Zhang *et al*., 1998; Cheng *et al*., 2002), C17-C21 (Matsuda *et al*., 1997; Melek *et al*., 2002; Waffo-Téguo *et al*., 2004) and C13-C17 (Ouyang *et al*., 1996), and probably reflects that the cyclases can differ in their mechanisms of facilitating hydride shifts. Single unsaturation at position C12-C13 is the most common. Double unsaturations were found at position C6-C7 and C12-C13 (Sahu, 1996), C5-C10 and C12-C13 (Sahu, 1996), C10-

C11 and C12-C13 (Sahu, 1996; Marx Young *et al*., 1997), as well as C11-C12 and C13-C17 (Sánchez-Contreras *et al*., 2000; Melek *et al*., 2002).

Sugar chains attached to skeleton (1) saponins

Saponins belonging to the same skeletal group can have a huge variation in the number and type of sugar residues attached. **Table 3** summarises some features of the various sugar chains that can be attached to different carbon atoms of skeleton (1). The number and type of sugar chains can vary enormously, *e.g*., there are 17 different 2-residue sugar chains and 26 different 3-residue sugar chains present in the Fabales alone. Therefore, the table only indicates the number of residues per chain and the number of different chains of that particular length for the relevant carbon atoms, for each plant order. Short sugar chains are usually attached at C2 and/or C18 and they have a length of 1-8 residues. There are a few cases in which sugar chains are attached at C1, C14, C19 and C20, and these are short chains of 1 or 2 residues only. The sugar chain at C14 occurs only in the plant order of Sapindales (Elgamal *et al*., 1995b), whereas the glycosylation at C19 (Mohamed *et al*., 1995; Mbafor *et al*., 1997; Gu *et al*., 2002; Decroos *et al*., 2005) and C20 (Mahato *et al*., 1992b; Woldemichael & Wink, 2002; Woldemichael *et al*., 2003) occur only in the Fabales. The sugar attachment at C1 can be present in several plant orders, such as the Caryophyllales (Li *et al*., 1994a; Elgamal *et al*., 1995a), Ebenales (Wandij *et al*., 2003), Celastales (Amimoto *et al*., 1993) and Fabales (Oleszek *et al*., 1992). The Fabales contain the largest number of possible glycosylation sites, at C1, C2, C18, C19 and C20.

Most of the saponins are monodesmosides or bidesmosides, which means that they contain either 1 or 2 sugar chains, respectively, at different carbon positions. There are 2 exceptions to this; an alfalfa (Oleszek *et al*., 1992) and an *Acacia auriculiformis* saponin (Mahato *et al*., 1992b), which are tridesmoside saponins. The sugar chains of skeleton (1) are often branched and can consist of up to 8 sugar residues, commonly, glucose, arabinose, rhamnose, xylose and glucuronic acid (Haralampidis *et al*., 2002). A few less common sugar residues are also found, such as apiose, fucose, quinovose, allose and ribose (**Table 3**). Apiose and fucose are distributed in many plant orders.

Table 3: Sugar chains attached to skeleton (1) in different plant orders

Plant subclasses	Plant orders	Number of residues per chain/Number of different chains C1(*4*)*	C2(*3*)	C14	C18(*17*)	C19(*22*)	C20(*21*)
Class of Magnoliopsida							
Asteridae	Asterales[api, fuc]		**1**3, **2**6, **3**4, **4**3		**1**3, **2**3, **3**2, **4**2, **5**2, **6**1		
	Dipsacales[all]		**1**2, **2**2, **3**4, **4**2, **5**2, **6**1, **7**2, **8**1		**1**1, **2**3, **3**1		
	Gentianales		**1**1, **2**1, **3**1		**1**1, **2**1		
	Lamiales[api]		**1**1, **2**1, **3**1		**1**1, **3**1, **4**1, **5**1		
	Rubiales[api, fuc]		**1**5, **2**9, **3**3		**1**1, **2**2, **3**2, **4**1		
	Scrophulariales[fuc]		**1**2, **2**6, **3**1, **4**1		**1** 1		
Caryophyllidae	Caryophyllales[fuc, qui]	**1** 1	**1**4, **2**12, **3**10, **4**3, **6**1		**1**2, **2**1, **3**2, **4**6		
Dilleniidae	Ebenales[api]	**1** 1	**1**2, **2**3, **3**3, **4**1		**1**1, **2**1, **4**2, **5**1, **6**1		
	Lecythidales		**3** 1		**1** 1		
	Primulales[fuc]		**1**1, **3**3, **4**3, **5**5		**3**1, **5**2		
	Theales		**2** 1				
	Violales				**1** 1		
Magnoliidae	Ranunculales[rib]		**1**2, **2**5, **3**7, **4**1		**1**1, **2**1, **3**2		
Rosidae	Apiales[fuc]		**1**4, **2**13, **3**20^{S}, **4**1		**1**2, **2**3, **3**1, **4**1, **5**1		
	Celastales	**1** 1	**1**3, **2**2, **3**4		**1**1, **2**1, **3**1		
	Fabales[api, fuc]	**1** 1	**1**6, **2**17, **3**26, **4**1, **6**1		**1**2, **2**1, **3**5, **4**5, **5**3	**1**2, **2**3	**1**1, **2**1
	Myrtales		**1** 1		**1** 1		
	Polygalales[fuc]		**1** 1		**4**1, **5**1		
	Rosales[api, fuc, qui]		**1**3, **2**1, **3**4, **4**1		**4**1, **5**1		
	Santalales		**2**1, **3**2		**1** 1		
	Sapindales[api, fuc, qui]		**1**6^{S}, **2**9, **3**17, **4**3	**1** 1	**1**1, **2**3, **3**1, **4**1, **5**1		
Class of Liliopsida							
Commelinidae	Cyperales		**3** 2				
Liliidae	Liliales[api, fuc]		**1**3, **2**1		**2**1, **3**5, **4**1		

Api, fuc, qui, all and rib represent apiose, fucose, quinovose, allose and ribose, respectively, which are the less common sugars that can be present in the sugar chains.

"*S*" indicates that a sugar unit is sulphated.

* Number within bracket indicates IUPAC numbering

Apiose is present in the Asterales (Su *et al*., 2001), Lamiales (Burger *et al*., 1998), Rubiales (Gariboldi *et al*., 1990), Ebenales (Li *et al*., 1994b; Charrouf *et al*., 1992; Nicolas *et al*., 1995), Fabales (Oleszek *et al*., 1992; Sakai *et al*., 1999), Rosales (Guo & Kenne, 2000; Guo *et al*., 2000; Nyberg *et al*., 2000), Sapindales (Jayasinghe *et al*., 1995; Voutquenne *et al*., 2003) and Liliales (Asada *et al*., 1994), whereas fucose is present in Asterales (Shao *et al*, 1995; Schöpke *et al*., 1995; 1996; 1997), Rubiales (Lamidi *et al*., 1995; Fang *et al*., 1995; He *et al*., 1996), Scrophulariales (Klimek *et al*., 1992), Caryophyllales (Schröder *et al*., 1993; M'Bark *et al*., 1996; Sang *et al*., 2000), Primulales (Zhang *et al*.,1998), Apiales (Ebata *et al*., 1996; Matsuda *et al*., 1997), Fabales (Beutler *et al*., 1997), Polygalales (Kuroda *et al*., 2001; Yui *et al*., 2001), Rosales (Guo *et al*., 2000; Nyberg *et al*., 2000), Sapindales (Lavaud *et al*., 1998) and Liliales (Asada *et al*., 1994). Quinovose, on the other hand, is more order specific and is present in the Caryophyllales (Sanoko *et al*., 1999), Rosales (De Tommasi *et al*., 1996) and Sapindales (Elgamal *et al*., 1995b; Pöllmann *et al*., 1997). Allose and ribose are only present in 1 plant order, the Dipsacales (Baykal *et al*., 1998) and the Ranunculales (Wang *et al*., 1997b), respectively.

The 3-residue sugar chains are the most diverse, with more than 100 different chains occurring in all plant orders except the Theales (Lu *et al*., 2000), Violales (Dijoux *et al*., 1993), Myrtales (de Carvalho *et al*., 1999) and Polygalales (Kuroda *et al*., 2001) (**Table 3**). The plant order of Dipsacales shows the largest diversity in chain length, from 1 to 8 sugar residues, which are attached at C2 (Ma *et al*., 1992). Theales, on the other hand, has only a 2-residue sugar chain at C2 and Violales has only a 1-residue sugar chain at C18. The class of Liliopsida seems to be less diverse than that of Magnoliopsida with respect to glycosylation patterns. They generally have shorter sugar chains and there is little variation in the type of sugar residues. It was observed that most of the saponins that contain a 1-residue sugar chain at one carbon position usually have a longer sugar chain at another position (Ouyang *et al*., 1997; Schöpke *et al*., 1997; Burger *et al*., 1998; Shaker *et al*., 1999; Treyvaud *et al*., 2000; Kuroda *et al*., 2001; Woldemicheal & Wink, 2002). This might suggest that a short sugar chain needs to be compensated by a longer one in order to keep saponin molecules water-soluble. However, this may not always be the case, as can be seen from *e.g*., a saponin from *Aphloia Madagascariensis*, Violales (Dijoux *et al*., 1993), which has only a sugar at C18, and a saponin from *Margyricarpus setosus*, Rosales (De Tommasi *et al*., 1996), which has only a sugar at C2. Saponins from *Zygophyllum* species, Sapindales (Pöllmann *et al*., 1997) and *Lafoensia glyptocarpa*, Myrtales (de Carvalho *et al*., 1999) have only a sugar at C2 and/or C18. It was also noticed that sulphated sugar residues are usually present in short chains of 1 or 3 residues

(**Table 3**). Most of the longer chains (4-8 residues) contain no glucuronic acid, whereas many of the short chains (3 or less residues) contain glucuronic acid. These observations support the hypothesis that charged sugar residues make saponins more water-soluble, and that their absence might be compensated by longer sugar chains. Furthermore, it was observed that the longer sugar chains (4 to 8 residues) are plant order specific, with 1 exception, the sugar chain of rha(1→3)xyl(1→4)rha(1→2)ara(1→**18**), which is present in 3 plant orders, Lamiales (Burger *et al*., 1998), Rubiales (Gariboldi *et al*., 1990) and Ebenales (Li *et al*., 1994b; Sahu *et al*., 1997) (**Table 3**). It has been reported that all saponins have a sugar chain attached to the C2 position of their aglycones as a common feature (Haralampidis *et al*., 2002). This may be true for most saponins but not for all, because *e.g*., the saponins from *Acanthopanax spinosus* (Miyakoshi *et al*., 1993), *Mussaenda pubescens* (Zhao *et al*., 1996; 1997), *Erythrina sigmoidea* (Mbafor *et al*., 1997), *Vaccaria segetalis* (Yun *et al*., 1998), *Acanthopanax japonicus* (Park *et al*., 2002) and *Aphloia Madagascariensis* (Dijoux *et al*., 1993) do not have a sugar chain attached at C2.

Concluding remarks

By considering the biosynthetic origin of the various saponins and simplifying their structures by omitting (part of the) substituents that were added after cyclization of oxidosqualene, we have been able to attribute the various saponins that have been described in the literature to five main classes. It was observed that saponin biosynthesis does not seem to be plant order specific, not only with respect to the cyclization of oxidosqualene to the various skeletons, but also with respect to post-cyclization events, such as decoration of the skeletons with various functional groups and (with a few exceptions) glycosyl residues. However, a much clearer understanding of how the various saponin structures relate to each other has been obtained, which was strengthened by our observations that, within one main class, certain skeletons and their precursors consistently appeared in the various plant orders. The five main classes of saponins established in this review may serve as a stepping-stone for further classification of new saponins that will be isolated in the future. It can also possibly assist in defining target genes in saponin biosynthesis studies for the various cyclases, particularly for the many enzymes acting down-stream in the saponin biosynthesis pathway.

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Chapter 5

Presence and release of volatile organic compounds from various pea protein preparations (*Pisum sativum* L)

ABSTRACT

The volatile organic compounds (VOCs) such as aldehydes, ketones and alcohols released from pea flour, protein isolate, legumin and vicilin at pH 8, were identified and quantified using dynamic headspace, gas chromatography (GC) and GC-mass spectrometry. This study gives an overview of the amount and type of VOCs present in pea flour and its protein preparations. The VOCs released differed in concentration between the various protein preparations. Hexanal was the major VOC released from pea flour and the protein isolate, whereas 2-ethyl-1-hexanol was the major VOC released from the legumin and vicilin preparations. In addition to pH, protein purification had a large effect on the amount and type of VOCs released. The presence of various VOCs in the various preparations can be largely explained by their log P values; their high extractability with aqueous solvents at low log P and their enrichment in the purified fractions at high log P.

KEYWORDS: *Pea flour, volatile organic compound(s), protein isolate, legumin, vicilin, purification, pH, log P*

INTRODUCTION

Proteins from plant sources, such as pea proteins, are well suited to be used as ingredients to formulate new food products, *e.g*. Novel Protein Foods (NPFs) (Davies & Lightowler, 1998; Zhu *et al*., 2004). The use of plant protein ingredients may cause modifications in the overall flavour perception, by reducing the aroma compound intensities or via their endogenous flavours (Plug & Haring, 1993; 1994).

Peas are generally regarded as a good source of protein because of their relatively high protein content (Reichert & MacKenzie, 1982). Pea protein consists mainly of the salt-extractable globulins (legumin and vicilin), which account for 65-80% of the extractable proteins (Derbyshire *et al*., 1976). Pea legumin is a protein of ~60 kDa that consists of a basic 20 kDa and an acidic 40 kDa polypeptide linked together by a disulphide bridge. It assembles as a hexamer (360 kDa) at pH 7-9 (Guéguen *et al*., 1988). Vicilin is a protein of ~50 kDa which assembles into a trimer *in vivo* (Gatehouse *et al*., 1981). Pea proteins, like other legume proteins, may be used as a crude meal, a protein concentrate or a protein isolate. The fractionation steps to prepare these protein preparations, *e.g*. aqueous extraction and precipitation at acidic pH, may cause the removal or enrichment of certain volatile organic compounds (VOCs). The binding of diacetyl to pea proteins, for example, was observed to decrease with decreasing pH within the range of 9 to 2 (Dumont & Land, 1986). Proteins such as caseins, bovine serum albumin and β-lactoglobulin, have also been shown to adsorb less VOCs at acidic pH than at basic pH (Druaux *et al*., 1995; Le Thanh *et al*., 1992; Andriot *et al*., 1999; Rogacheva *et al*., 1999). These studies thus show that treatments such as pH changes may have a significant influence on the adsorption of VOCs to proteins.

Many VOCs have been identified in peas (*Pisum sativum* L), which include several groups of potential flavour compounds such as alcohols, aldehydes, ketones and esters (Murray *et al*., 1976; Jakobsen *et al*., 1998). Identification and quantification of the types of VOCs in pea flour and protein preparations at various pHs is, therefore, of significant importance for the utilisation of pea-derived materials as food ingredients. However, up till now, such information is lacking.

The aim of this study was to identify and quantify the release of VOCs from the various protein preparations and to study the effect of pH and degree of purification on the release of these VOCs.

MATERIALS AND METHODS

Protein purification

Dried de-hulled split green peas (*Pisum sativum L, Solara spp.*) that were grown commercially in the Netherlands were supplied by Cebeco (Vlijmen, The Netherlands). Legumin and vicilin were purified using a procedure designed from the methods of Thomson *et al*. (1978), Koyoro & Powers (1987) and Bora *et al*. (1994). The steps involved in the purification process include extraction, precipitation and anion exchange chromatography (**Figure 1**). Green peas were milled in a commercial blender (Waring, New Hartford, Connecticut, U.S.A.) in a ratio 1:1 with dry ice (w/w), to avoid possible heat denaturation of the proteins. Legumin and vicilin extract was obtained by extracting pea flour with 100 mM Tris-HCl buffer (1:10 (w/v)) pH 8 for 2 h at room temperature. This crude protein extract was centrifuged at 12,300 x *g* at 10 ^{o}C for 30 min. The supernatant collected was precipitated at pH 4.8 after adjusting the pH with 1 M HCl, to isolate the globulins. The precipitated protein isolate was collected after centrifugation at 36,000 x *g* at 4 °C for 25 min. The protein pellet (protein isolate) was washed with water (pellet to water ratio 1:10 (w/v)) to remove any of the water-soluble albumins, followed by centrifuging at 36,000 x *g* at 4 °C for 25 min. The protein isolate collected was then suspended in 100 mM Tris-HCl buffer at pH 8.0 (1:20 (w/v)) with continuous stirring for 2 h followed by centrifugation at 36,000 x *g* at 4 °C for 25 min. The supernatant obtained was dialysed at 4 °C against McIlvaine buffer at pH 4.8 (0.2 M Na_2HPO_4 + 0.1 M citric acid) containing 0.2 M NaCl (supernatant to buffer ratio of 1:20 (v/v)). The dialysis buffer was changed three times over a period of 24 h. This yielded a protein-rich precipitate. After the 24 h period, the dialysed sample was centrifuged at 36,000 x *g* at 4 °C for 25 min to separate the precipitated protein from the supernatant. The precipitated protein was freeze-dried and was referred to as 'protein enriched fraction'. The protein enriched fraction was re-dissolved by stirring for 2 h in 35 mM potassium phosphate buffer, pH 7.6, containing 0.075 mM NaCl (buffer A), at a protein concentration of 25 mg/mL, followed by centrifugation at 36,000 x *g*, at 4 °C for 25 min. The supernatant collected after centrifugation was filtered through a membrane filter with a pore size of 0.2 μm (Schleicher & Schuell, Keene, NH, USA). A DEAE Sepharose Fast Flow column (10 x 15 cm; Pharmacia Biotech, Uppsala, Sweden) previously rinsed with 6 volumes of millipore water (~1.8 L), followed by 6 volumes of buffer A, was used for purifying the supernatant. Approximately 1200 mL of the filtered supernatant was applied onto the DEAE column at a flow rate of 15 mL/min, followed by rinsing with 1 volume of buffer A. Elution was performed with a linear salt gradient of 0.075-0.5 M NaCl over 6 column volumes at a flow rate of 30 mL/min.

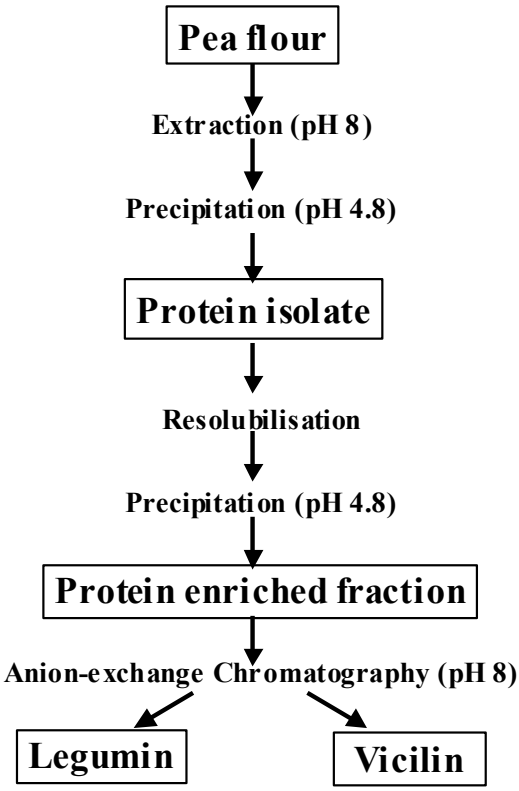

Figure 1: Flowchart of legumin and vicilin purification

Using UV detection at 280 nm, 15 mL fractions were collected and appropriate fractions were subsequently analysed with SDS-PAGE (Bio-Rad ready gel, 10-20% Tris-HCl, Coomassie blue staining). Fractions containing only vicilin or legumin were pooled. The 2 pools of fractions obtained were dialysed against distilled water, freeze-dried and stored at –20 °C. These freeze-dried materials (legumin and vicilin) were used for the preparation of protein solutions. The protein concentrations of legumin and vicilin solutions (0.1% w/v) were determined using the Bradford protein assay (Bradford, 1976) using bovine serum albumin (BSA) (Sigma Aldrich, Steinheim, Germany) as a standard.

Dynamic headspace sampling (purge and trap)

Dynamic headspace sampling was used for the collection of headspace VOCs from the pea flour, crude protein isolate, legumin and vicilin dispersions. Dry samples (100 mg) of de-hulled pea flour, crude protein isolate, legumin or vicilin were dispersed in 10 mL of McIlvaine buffer (0.2 M Na_2HPO_4 + 0.1 M citric acid) at pH 4 (only pea flour) or pH 8 followed by the addition of 200 µL

of 3.5% (v/v) of an anti-foaming agent (silicon SAG 30; A. Smit & Zoon b.v., Weesp, The Netherlands) to prevent foaming of protein solution during purging. These mixtures were subsequently stirred for 12 h in an airtight glass vessel at 4 ^{o}C to ensure complete sample hydration. Dynamic headspace sampling was performed with the sample in the vessel, at 37 ^{o}C in a water bath for 1 h with constant purging of nitrogen gas at a flow rate of 45 mL/min. VOCs released from the samples were entrapped on 0.1 g Tenax TA (poly-2,6-diphenyl-p-phenylene oxide), 35/60 mesh (Alltech Nederland b.v., Zwijndrecht, The Netherlands) in a glass tube (3 x 100 mm).

Analysis of volatiles

VOCs entrapped in the Tenax tube were analysed by thermal desorption (245 ^{o}C, 5 min) followed by cold trap (-120 ^{o}C) and subsequent evaporation (240 ^{o}C) using a Carlo Erba TDAS 5000 device (Interscience b.v., Breda, The Netherlands). The components were analysed by gas chromatography (GC) on a Supelcowax 10 capillary column (Supelco Inc., Bellefonte, PA) (60 m long, 0.25 mm i.d. and 0.25 μm film thickness) using a Carlo Erba MEGA 5300 gas chromatograph (Interscience b.v., Breda, The Netherlands) equipped with a flame ionisation detector (FID) heated at 275 ^{o}C. The oven temperature programme started at 40 ^{o}C (4 min hold), followed by heating to 92 ^{o}C at a rate of 2 ^{o}C/min, and then heating to 272 ^{o}C at a rate of 6 ^{o}C/min with a final hold of 5 min at 272 ^{o}C. Identification of VOCs was performed by gas chromatography-mass spectrometry (GC-MS) using a Varian 3400-Finnigan MAT 95 instrument equipped with a thermal desorption/cold trap device (TCT injector 16200, Chrompack). Mass spectra were recorded at an electron impact ionisation mode of 70 eV and a scan of m/z = 24 to 300 sec/decade with a cycle time of 0.97 sec. GC conditions for GC-MS were the same as in GC-FID analysis. Identification of compounds was done on the basis of an MS database and by comparison of retention times.

RESULTS

Table 1 and **Table 2** show the volatile organic compounds (VOCs) released from pea flour at **pH 4 and pH 8**, and from several pea protein preparations at **pH 8**. The VOCs were classified into 4 groups. The first three groups of compounds, aldehydes, ketones and alcohols, are commonly found in green peas (Murray *et al*., 1976; Jakobsen *et al*., 1998). Compounds that do not belong to any of these 3 groups were placed in a 4th group, which was referred to as 'others'. The VOCs within each group were sorted according to increasing log P values, which is commonly used to describe hydrophobicity of VOCs.

VOCs released from pea flour at various pH

It can be seen in **Table 1** that aldehydes were the major group of VOCs released from pea flour at both pHs. At pH 8, a total of 21 different VOCs were released, of which 9 were aldehydes, 2 were ketones, 9 were alcohols and 1 other. A total of 18 different VOCs were released at pH 4, of which 7 were aldehydes, 4 were ketones, 6 were alcohols and 1 other. The total amount of volatiles released (expressed in arbitrary units; AU), per gram of dry matter, was higher at pH 8 than at pH 4.

From **Table 1**, it can be seen that the aldehyde, hexanal was the major volatile compound released from pea flour at both pH 4 and pH 8. It represented >35% of the total amount of volatiles released (the response factors of all aldehydes are similar). The lower aldehydes (pentanal and 2-hexenal) and the lower alcohols (2-propanol and 1-penten-3-ol) were only released at pH 8. On the other hand, decanal, undecanal, 1-penten-3-one and acetophenone were only released at pH 4. The amounts of aldehydes and alcohols released from pea flour were observed to be higher at pH 8 than at pH 4.

Table 1: Volatile organic compounds detected in pea flour at pH 4 and pH 8

Pea flour	Log P*	pH 4 [a]	pH 8 [a]
Aldehydes			
3-methylbutanal	1.25	+	+
pentanal	1.44	-	+++
2-Hexenal (E)	1.58	-	+++
hexanal	1.97	+++++	+++++
2-heptenal (E)	2.11	-	+++
heptanal	2.50	+	+
2-octenal (E)	2.64	-	++
2-nonenal (E)	3.17	+	+
nonanal	3.56	+++	+++
decanal	4.10	+++	-
undecanal	4.63	+	-
Total aldehydes (AU)		**199110**	**355510**
Ketones			
2-butanone	0.37	+	+
1-penten-3-one	0.57	+	-
acetophenone	1.67	+++	-
6-methyl-5-hepten-2-one	2.09	++	+++
Total ketones (AU)		**25900**	**19690**
Alcohols			
2-propanol	0.16	-	+
1-penten-3-ol	1.05	-	+++
2-penten-1-ol (E)	1.22	-	+
1-pentanol	1.41	+	+++
3-hexen-1-ol (Z)	1.61	-	+
1-hexanol	1.94	+++	+++
1-heptanol	2.47	+	-
1-octen-3-ol	2.64	-	++
2-methyl-1-propanol	2.64	+	+
2-ethyl-1-hexanol	2.82	+	++
1-octanol	3.00	++	-
Total alcohols (AU)		**28200**	**98690**
Others			
toluene	2.68	+	-
1-tetradecene	7.68	-	+
Total others (AU)		**2590**	**3500**
Total number of volatiles		**18**	**21**
Total volatiles (AU)		**255800**	**477390**

*Calculated using Advanced Chemistry Development (ACD) software solaris V4,67 (www.acdlabs.com) (1994-2004 ACD)

[a]Calculated as amount of volatile organic compounds in arbitrary units (AU) per g of dry matter

"+" represents the amount of volatiles between 500-5000 AU

"++" represents the amount of volatiles between 5000-10000 AU

"+++" represents the amount of volatiles between 10000-50000 AU

"++++" represents the amount of volatiles between 50000-100000 AU

"+++++" represents the amount of volatiles between 100000-500000 AU

VOCs present in different pea protein preparations

Figure 1 shows the steps involved in the preparation of pea protein isolate, legumin and vicilin, which include extraction, precipitation and anion exchange chromatography. The VOCs released from these various protein preparations are presented in **Table 2**. It can be seen that a total of 38 different VOCs were released from the pea protein preparations at pH 8. From pea flour 21 different VOCs were released, whereas 10 different VOCs were released from the protein isolate. From the legumin preparation 23 VOCs were released, whereas 18 VOCs were released from the vicilin preparation. The total amount of volatiles released per gram of dry matter, was the highest in the pea flour, followed by the legumin preparation, the vicilin preparation and the protein isolate.

Just as from pea flour, hexanal was also the major VOC released from pea protein isolate at pH 8 (**Table 2**), representing >34% of the total amount of volatiles released. The amount of hexanal released, per gram of dry matter, decreased drastically (>90%) when going from pea flour to the protein isolate, and decreased further when going from the protein isolate to the legumin and vicilin preparations. Most of the other aldehydes (*e.g*. 2-hexenal, 2-heptenal) were already removed during extraction/precipitation. The concentration of *e.g*. pentanal was greatly reduced (> 90%) when going from the pea flour to the protein isolate, before being completely removed from the legumin and vicilin preparations. 2-Nonenal and undecanal seemed to be enriched in the legumin and vicilin preparations. All ketones were enriched in both the legumin and vicilin preparations. Acetophenone and 6-methyl-5-hepten-2-one were especially abundant in the legumin preparation, whereas 2-heptanone was more abundant in the vicilin preparation. Most of the alcohols present in pea flour were already removed during extraction/precipitation, causing the pea protein isolate to contain only a few VOCs. 2-Ethyl-1-hexanol was present in all 4 preparations and was considerably enriched in the legumin and vicilin preparations, being the major VOC released from these preparations. Decane was particularly enriched in both the legumin and vicilin preparations.

Table 2: Volatile organic compounds detected in pea flour and protein fractions at pH 8

pH 8	Log P*	Pea flour[a]	Protein Isolate[a]	Legumin[a]	Vicilin[a]
13 Aldehydes					
3-methylbutanal	1.25	+	-	-	+
2-pentenal (E)	1.04	-	-	+	-
pentanal	1.44	+++	+	-	-
2-Hexenal (E)	1.58	+++	-	-	-
hexanal	1.97	+++++	+++	+	+
2-heptenal (E)	2.11	+++	-	-	-
heptanal	2.50	+	-	++	-
2-octenal (E)	2.64	++	-	-	-
octanal	3.03	-	+	+	-
2-nonenal (E)	3.17	+	-	++	++
nonanal	3.56	+++	-	-	-
decanal	4.10	-	-	+	-
undecanal	4.63	-	-	+	+
Total aldehydes (AU)		**355510**	**18142**	**26669**	**11926**
5 Ketones					
2-butanone	0.37	+	+	-	+
acetophenone	1.67	-	-	++	+
2-heptanone	1.97	-	-	+	+++
6-methyl-5-hepten-2-one	2.09	+++	-	+++	++
2-decanone	3.56	-	+	+	-
Total ketones (AU)		**19690**	**1598**	**22279**	**26413**
13 Alcohols					
2-propanol	0.16	+	+	-	-
1-butanol	0.88	-	-	+	-
1-penten-3-ol	1.05	+++	-	+++	+++
2-penten-1-ol (E)	1.22	+	-	-	-
1-pentanol	1.41	+++	-	+	-
3-hexen-1-ol (Z)	1.61	+	-	-	-
1-hexanol	1.94	+++	-	-	-
1-heptanol	2.47	-	-	-	+
1-octen-3-ol	2.64	++	-	-	-
2-methyl-1-propanol	2.64	+	-	+	+
2-ethyl-1-hexanol	2.82	++	+	+++++	++++
1-octanol	3.00	-	+	+	+
1-nonanol	3.53	-	-	+	+
Total alcohols (AU)		**98690**	**3855**	**256421**	**140052**
7 Others					
toluene	2.68	-	+	+	+
2-pentylfurane	3.97	-	-	+	-
2,4,4-trimethyl-1-pentene	4.15	-	-	+	-
1-Octene	4.50	-	-	-	+
limonene	4.58	-	-	+	-
decane	6.10	-	-	+++	+++
1-tetradecene	7.68	+	+	-	+
Total others (AU)		**3500**	**2392**	**21855**	**24243**
Total number of volatiles	**38**	**21**	**10**	**23**	**18**
Total volatiles (AU)		**477390**	**25987**	**327224**	**202634**

*Calculated using Advanced Chemistry Development (ACD) software solaris V4,67 (www.acdlabs.com) (1994-2004 ACD)

[a]Calculated as amount of volatile organic compounds in arbitrary units (AU) per g of dry matter

"+" represents the amount of volatiles between 500-5000 AU

"++" represents the amount of volatiles between 5000-10000 AU

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DISCUSSION

The VOCs in this study differ markedly in their log P values. The log P value is often considered as an important characteristic of VOCs as it represents the hydrophobicity of the compound. Hydrophobic compounds, *i.e*. compounds with a relatively high log P value, generally bind more strongly to proteins and are not easily extracted with aqueous solvents.

Flavour release in relation to pH

From **Table 1**, it can be seen that the VOCs (both aldehydes and alcohols) that were only released at pH 4 (*e.g*. decanal, undecanal, 2-ethyl-1-hexanol, 1-octanol) generally had high log P values (>3), whereas many of those that were only released at pH 8 (*e.g*. pentanal, 2-hexenal, 2-propanol, 1-penten-3-ol) were more polar (log P values <2). The solubility of the proteins in pea flour differs with pH, being higher at pH 8 than at pH 4 (Derbyshire, 1976; Lasztity *et al*., 1986). The release of VOCs with a low log P value at pH 8 could therefore be due to the fact that pea flour at that pH becomes more easily accessible to the aqueous extraction solvent due to the solubilisation of the proteins, resulting in an increased rate of release of the more polar VOCs.

Effect of purification steps

The steps involved in the pea protein purification process (extraction/precipitation and anion-exchange chromatography) were shown to have a significant effect on the quantity and type of VOCs present in the protein preparations.

(i) Extraction and precipitation at pH 4.8

Aqueous extraction led to a major reduction in the release of aldehydes, ketones and alcohols. Of the major components that are being removed from pea flour by aqueous extraction, hexanal is reported as a major breakdown product or derivative that is frequently formed during the oxidation of fatty acids by lipoxygenase (Lumen *et al*., 1978; Sessa, 1976). It was present in large quantities in blanched green peas, representing about 55% of the total trapped volatiles (Jakobsen *et al*., 1998). Its presence is of importance because it possesses a strong odour and is responsible for the "hay-like" off-flavour in peas (Murray *et al*., 1976; Bengston and Bosund, 1964; Williams *et al*., 1986).

During the extraction of pea flour and the subsequent precipitation, several VOCs were either completely removed, or their concentrations were reduced (**Table 2**). VOCs such as 2-hexenal, 2-penten-1-ol, 3-hexen-1-ol and 1-hexanol (**Table 2**), which have relatively low log P values of <2, seemed not to be present anymore in the protein preparations. These VOCs are relatively polar and are, therefore, presumably easily extracted with aqueous solvents and are not precipitated with the proteins. On the other hand, VOCs such as undecanal, 2-decanone, 1-nonanol and decane with log P values of >3, were found to be enriched in the protein preparations. These apolar VOCs are probably not extracted as such, but are retained strongly by the proteins, and thus enriched during protein precipitation.

(ii) Purification: Enrichment in protein fractions

Alcohols were found to be substantially enriched in the legumin and vicilin preparations, compared to the protein isolate (**Table 2**), *e.g*. 2-ethyl-1-hexanol increased by more than 30-fold and 15-fold when going from pea flour to the legumin and the vicilin preparations, respectively. These alcohols are known to possess strong odours (Murray *et al*., 1968) and may thus influence final flavour perception. The absence of some alcohols (*e.g*. 2-penten-1-ol, 3-hexen-1-ol, 1-hexanol and 1-octen-3-ol) in the protein preparations may be due to the removal of these alcohols during extraction/precipitation. Some of the other VOCs, such as decanal, undecanal, 2-decanone, 1-nonanol and decane, were not detected as being released from the pea flour, but were apparently very much enriched during the purification process that could be detected as being released from either the protein isolate or the legumin or vicilin preparations (**Table 2**). These VOCs probably have a relatively high affinity for proteins, resulting in their enrichment during protein purification. The higher concentrations of total volatiles present in the legumin preparation compared to the vicilin preparation may also indicate that legumin in general has a higher affinity for VOCs than vicilin.

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Chapter 6

Interaction of pea vicilin with volatile organic compounds

This Chapter is based on Lynn Heng, G.A. van Koningsveld, H. Gruppen, M.A.J.S. van Boekel, J-P. Vincken, J.P. Roozen, A.G.J. Voragen. Protein-flavour interactions in relation to development of novel protein foods. *Trends Food Sci. Technol*., 2004, 15, 217-224.

ABSTRACT

The interactions between volatile organic compounds (VOCs) and pea vicilin (7S) and the effect of heat treatment on these interactions were studied. At pH 7.6, vicilin was found to interact with both aldehydes and ketones, as measured with static headspace-gas chromatography. Heating of vicilin at 90 ^{o}C resulted in decrease interactions of these VOCs with the protein. Vicilin preparations were adjusted to pH 4.5, 5.5 and 6.5, which resulted in the formation of a soluble and an insoluble vicilin fraction. The insoluble fractions showed remarkably high affinities for octanal than the supernatant fractions, at the same pH. At all pHs, the insoluble fraction contained relatively high amounts of lipids and carbohydrates. After extraction with various organic solvents, the pellets at pH 4.5 and 6.5 showed a decrease in lipid and carbohydrate contents. These decreases, probably due to galactolipids, resulted only in a partial removal of the compounds interacting with octanal.

KEYWORDS: *Pea(s), vicilin, protein-flavour interaction, heat, lipid(s), carbohydrate(s)*

INTRODUCTION

Proteins may influence flavour perception of food through the interaction with volatile organic compounds (VOCs) (Martens *et al*., 1987). In recent years, protein sources from leguminous plants such as peas and soybeans, have been used to formulate new products, *e.g*., Novel Protein Foods (NPFs) (Davies & Lightowler, 1998). It is important that the taste and aroma of these protein foods are appealing and acceptable to consumers.

Several studies have shown that VOCs interact with proteins such as soy proteins (Arai *et al*., 1970; Damodaran & Kinsella, 1981a/b; Aspelund & Wilson, 1983; O'Neill & Kinsella, 1987a; Chung & Villota, 1989; O'Keefe *et al*, 1991a/b) and milk proteins (O'Neill & Kinsella, 1987b; 1988; Andriot *et al*., 1999; Rogacheva *et al*., 1999; Guichard & Langourieux, 2000). These interactions are influenced by parameters such as pH, temperature and ionic strength (Andriot *et al*., 1999; O'Neill & Kinsella, 1988; Guichard & Langourieux, 2000). Protein-flavour interactions were *e.g*. observed to be favoured at alkaline pH (Andriot *et al*., 1999; Rogacheva *et al*., 1999; Guichard & Langourieux, 2000). Thermal denaturation of proteins may either increase (Arai *et al*., 1970; Damodaran & Kinsella, 1981a; O'Neill & Kinsella, 1988) or decrease (Chung & Villota, 1989) their interactions with VOCs. Addition of chaotropic salts often reduces VOCs interactions whereas, lyotropic salts seem to enhance these interactions (Andriot *et al*., 1999; Guichard & Langourieux, 2000), indicating that hydrophobic interactions dominate in these interactions. Also, protein isolates, *e.g*. those from peas, may contain non-protein components such as lipids and carbohydrates, which are known to interact with significant amounts of VOCs (Godshall, 1997; Plug & Haring, 1993; 1994; Guichard, 2002). Therefore, if present, these components may influence the interactions of proteins with odour active compounds.

Peas contain 2 major protein fractions, legumin and vicilin, which are salt-extractable globular proteins that account for 65-80% of the total amount of proteins in peas (Owusu-Ansah & McCurdy, 1991). Pea legumin is a protein of ~60 kDa that consists of a basic 20 kDa and an acidic 40 kDa polypeptide, interlinked by a disulphide bridge, which assemble into a hexamer (360 kDa) at pH 7-9 (Guéguen *et al*., 1988). Vicilin is produced as a precursor of ~50 kDa and it assembles into a trimer *in vivo* (Gatehouse *et al*., 1981). Only a few VOC interactions studies have been performed with pea proteins (Dumont, 1985, Dumont & Land, 1986). The interaction of diacetyl, a ketone, to pea protein isolates was studied in the pH range of 2-9 (Dumont & Land, 1986). The free

ketone concentration was shown to decrease at higher pHs. No study has yet been performed on the interactions of the individual pea proteins with VOCs. Therefore, the aim of this study was to investigate the interactions between pea vicilin and VOCs, and the effect of heat treatment on these interactions.

MATERIALS AND METHODS

Purification of vicilin

Vicilin was purified from green peas (*Pisum* s*ativum, Solara spp.*) obtained from Cebeco (Vlijmen, The Netherlands) using a procedure adapted from the methods of Koyoro & Powers (1987) and Bora *et al*. (1994). Green peas were mixed 1:1 with dry ice (w/w) and milled in a commercial blender (Waring, New Hartford, Connecticut, U.S.A.), to avoid heat denaturation of the proteins. A vicilin extract was prepared by extracting pea flour with 10 mM Tris-HCl buffer (1:10 (w/v)) at pH 8 for 2 h at room temperature. The extract was centrifuged at 12,300 x *g* at 10 °C for 30 min. The clear supernatant (~4.5 L) was collected and applied batch-wise to DEAE CL-6B Sepharose Fast Flow (Amersham Pharmacia Biotech, Uppsala, Sweden). Before use, the ion-exchange material was placed on a glass filter (Emergo glass filter 26 D2, 120 mm D50), rinsed with 6 volumes of millipore water (~1.8 L), followed by 6 volumes of 35 mM potassium phosphate buffer (pH 7), containing 0.075 mM NaCl. The equilibrated DEAE material was then mixed with 1.5 L of the clear supernatant in a glass beaker and gently stirred for two hours at 4 °C to allow protein binding. Next, the mixture was transferred to the glass filter again and rinsed with two volumes of the buffer (~0.6 L) to remove unbound material. The vicilin fraction was eluted with a 35 mM potassium phosphate buffer (pH 7) containing 0.4 M NaCl. The eluted fraction was diluted with 35 mM potassium phosphate buffer (pH 7) to reduce the salt concentration for further purification using a DEAE Sepharose Fast Flow column (5 cm diameter, 343 mL column volume; Pharmacia Biotech, Uppsala, Sweden). Before using the DEAE, the ion-exchange column was rinsed with 6 volumes of millipore water (~1.8L), followed by 6 volumes of 35 mM potassium-phosphate buffer (pH 7) containing 0.075 mM NaCl. Elution was performed with a linear salt gradient of 0.075-0.5 M NaCl over 6 column volumes at 20 mL/min. The eluate was monitored at 280 nm and fractions (15 mL) under the peaks were collected. The composition of the protein fractions obtained was determined using SDS-PAGE gel (Bio-Rad ready gel, 10-20% Tris-HCl, Coomassie blue staining), before pooling and combining the vicilin containing fractions. The pooled fractions were dialysed against distilled water before freeze-drying and subsequently stored at –20 °C.

Protein content

Protein contents were measured using the Dumas combustion method, on a Thermo Quest NA 2100 nitrogen and protein analyser (Intersciences, The Netherlands) according to the instructions of the manufacturer. Samples (400 μL), both supernatants and pellets after re-suspension, were put in

aluminium sample cups and dried at 80 ^{o}C in an oven overnight. The dried samples were cooled down to room temperature before measuring their weight. The samples were analysed using *D*-methionine as an external standard. The protein content was calculated using 5.7 as nitrogen to protein conversion factor (Sosulski & McCurdy, 1987).

Gel permeation chromatography (GPC)

The apparent molecular weight of vicilin before and after heat treatment was estimated by gel permeation chromatography (ÄKTA Purifier 900, Amersham Pharmacia Biotech AB, Uppsala, Sweden) using a Superose 6 column (3.2 x 300 mm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Vicilin samples (1 mg/mL) of 50 μL were injected on to the column that was equilibrated with a 75 mM potassium phosphate buffer (I=0.188 M) at pH 7.6, and run with the same buffer at a flow rate of 0.05 mL/min. Detection was done at 280 nm. Compounds used for calibration were: aldolase (158 kDa), catalase (232 kDa), ferritin (440 kDa) and Blue dextran (2000 kDa).

Hexane and chloroform/methanol (1:1) defatting

Purified freeze-dried vicilin was defatted with either hexane or chloroform/methanol (1:1) (v/v) (Merck, Darmstadt, Germany) for 6 h by Soxhlet refluxing followed by overnight air-drying. The proteins were then used for solubility tests, fatty acid analysis, carbohydrate analysis and volatile organic compounds (VOCs) interaction studies.

Soluble and insoluble vicilin at various pHs

Samples (10 mL; 0.2% (w/v)) of vicilin solution were transferred to glass tubes and the pH of the solutions was adjusted to pH 4.5, 5.5, 6.5 with 1 M HCl, followed by 2 h of incubation at 4 ^{o}C. The solutions were then centrifuged at 1500 x *g* at 10 ^{o}C for 30 min. The supernatants were transferred to other glass tubes and the pellets were rinsed twice with 5 mL of water. The washed pellets were again centrifuged at 1500 x *g* at 10 ^{o}C for 30 min, drained off and dried under a stream of air overnight. The weight of the dry pellets was subsequently determined. These pellets, after re-suspension in 10 mM potassium phosphate buffer (10 mL; pH 7.6), and the supernatants were used for interaction studies with octanal (0.026 mM) and for the analysis of fatty acids and carbohydrates. Vicilin solution at pH 7.6 was used as reference.

Fatty acid analysis

Fatty acid analysis was done according to Bligh & Dyer (1959). To the dried vicilin pellets, 100 µL of an internal standard solution (0.83 mg/mL margarinic acid methylester in hexane), 1.5 mL methanol, 0.9 mL 1% (w/v) EDTA in water and 1 mL dichloromethane were added. The mixture was vortexed for 10 min, followed by centrifuging at 1500 x *g* at 20 ^{o}C for 10 min. The lower organic layer of the mixture was collected and transferred to a glass tube. Dichloromethane (2 mL) was added to the top layer and the mixture was vortexed for 2 min before centrifuging at 1500 x *g* at 20 ^{o}C for 10 min, after which the lower organic layer was again removed. This was repeated 3 times and the organic layers collected were combined and dried under nitrogen at 37 ^{o}C for 2 h. Next, 0.5 mL of a 10% (v/v) Boron trifluoride (~1.3 M) in methanol (Fluka, Sigma Aldrich, Steinheim, Germany) was added to the dried fractions and the mixtures were heated at 100 ^{o}C for 1 h in capped glass tubes. After heating, the samples were cooled in an ice bath. Pentane (2 mL; Merck, Darmstadt, Germany) and 2.5 M NaOH (1 mL) were added to the heated samples. The mixture was vortexed for 2 min followed by centrifuging at 1500 x *g* at 20 ^{o}C for 5 min. The organic top layer of the mixture was collected in a glass tube. The aqueous bottom layer of the mixture was mixed with 2 mL pentane and mixed for 2 min before centrifuging at 1500 x *g* at 20 ^{o}C for 5 min. This was repeated 3 times and the top layers collected were combined and dried under nitrogen at 37 ^{o}C for 2 h. 250 µL hexane (Merck, Darmstadt, Germany) was added to the dried fraction, of which 2 µL was injected into a DB-WAX capillary column (Supelco Inc., Bellefonte, PA) (30 m long, 0.53 mm i.d. and 1.0 µm film thickness) with helium as the carrier gas at a flow rate of 10 mL/min. Gas chromatography (GC) analysis was done using a Carlo Erba HRGC 5300 gas chromatograph (Interscience b.v., Breda, The Netherlands) equipped with a flame ionisation detector (FID) set at 275 ^{o}C. The oven temperature programme started at 80 ^{o}C and was increased to 160 ^{o}C at a rate of 15 ^{o}C/min using a linear gradient, followed by increasing it to 240 ^{o}C at a rate of 5 ^{o}C/min and keeping it constant at 240 ^{o}C for 8 min. The individual fatty acids identified and quantified were added to give the total amount of lipids presented in Table 3.

Carbohydrate analysis

Carbohydrate analysis was performed according to De Ruiter *et al*. (1992). To the dried vicilin pellets, 1 mL of 2 N HCl in dry methanol was added and the mixtures were heated at 80 ^{o}C for 16 h. The samples were evaporated to dryness before 0.5 mL of 2 N trifluoro acetic acid (TFA) in water was added and the samples were again heated at 121 ^{o}C for 1 h. The sugars released during this hydrolysis step were analysed using high-performance anion exchange chromatography (HPAEC)

with pulsed amperometric detection (PAD). HPAEC was performed using a ThermoQuest HPLC system with a Dionex ED40 PAD detector. Separation was performed on a Carbopac PA1 column (4 x 250 mm) with a flow rate of 1 mL/min. Gradient elution was done by mixing distilled water (A), 100 mM NaOH (B) and 1000 mM NaOAc (C) in 100 mM NaOH. Samples (20 μL) were injected and separated using the following elution program: 26 min isocratically at 85%A and 15%B followed by a linear gradient to 100%B in 7 min. Subsequently a linear gradient was started to 94%B and 6%C within 12 min, followed by a linear gradient to 70%B and 30%C in 33 min. The column was washed for 5 min with 100%C followed by a 15 min wash step with 100 mM NaOH and equilibrated for 15 min with a mixture of 85%A and 15%B. The individual sugars identified and quantified were added to give the total amount of carbohydrates presented in Table 3.

Studies on interactions between protein and volatile flavour compounds

(i) Preparation of solutions

All VOCs used were of analytical grade (Sigma Aldrich, Steinheim, Germany). The aldehydes, pentanal and octanal, and the ketones, 2-pentanone, 2-hexanone, 2-heptanone and 2-octanone were used for pea vicilin interaction studies. Potassium phosphate buffer (10 mM, pH 7.6, I=0.024 M) was used to prepare VOCs and protein solutions. Protein suspensions were stirred for 2 h at room temperature and filtered through 0.2 μm CA-S filters (Schleicher & Schuell, Dassel, Germany). The clear filtrate was used for interaction studies. Heating of samples was done by putting the capped vials into a water bath (GLF, Salm en Kipp b.v., Breukelen, The Netherlands) at 90 ^{o}C for 30 min. The aldehyde solutions were prepared at concentrations of 0.013 to 0.08 mM (1.6 to 6.5 ppm), whereas those of ketones were prepared at concentrations of 0.06 to 2.3 mM (8 to 205 ppm). These concentrations chosen are within the solubility ranges of the compounds used. The protein solutions were prepared at concentrations of 0.2 to 2% (w/v). The pH of solutions was adjusted with 1 M HCl or 1 M NaOH. Millipore water was used for all solutions.

(ii) Static headspace gas chromatography (SH-GC)

Static headspace sampling was used to study the interaction of VOCs with vicilin. 0.5 mL of the VOCs solution was added to 0.5 mL of the protein solution in headspace vials (Alltech associates Inc., 11.5 mL, 46 x 22.5 mm) and capped immediately. The final concentrations of aldehyde solutions were 0.006 to 0.04 mM (0.8 to 3.3 ppm), whereas those of ketones were 0.03 to 1.2 mM (4 to 103 ppm). The final concentrations of protein solutions were 0.1 to 1% (w/v). For blank samples, 0.5 mL of the volatile flavour compounds solution was added to 0.5 mL of potassium

phosphate buffer. The vicilin pellets at the various pH (as described above), after re-suspension, were analysed in the same way. All samples were prepared in triplicates. Samples (heated and non-heated) were then analysed by gas chromatography (GC) using a Carlo Erba MEGA 5300 gas chromatograph (Interscience b.v., Breda, The Netherlands) equipped with a flame ionisation detector (FID) heated at 225 ^{o}C and a Combi PAL autosampler (CIC analytics) that incubates the samples at 37 ^{o}C for 10 min with a constant agitation of 500 rpm. A sample volume of 1 mL was drawn from the headspace of the vial and injected into a Supelco DB-WAX capillary column (Supelco Inc., Bellefonte, PA) (30 m long, 0.54 mm i.d. and 1 μm film thickness) with helium as the carrier gas at a flow rate of 0.9 mL/min. The injector temperature was set at 200 ^{o}C at an injection speed of 100 μL/sec, using a pre and post injection delay of 500 msec. The oven temperature programme started at 80 ^{o}C and was increased to 110 ^{o}C at a linear rate of 3 ^{o}C/min.

RESULTS AND DISCUSSION

Interaction of pea vicilin with aldehydes and ketones

Figure 1 shows the amount of pentanal and octanal bound to vicilin at pH 7.6 for concentrations of 0.006-0.04 mM. It can be seen that at 0.025 mM of octanal, about 23 nmoles of octanal were bound per mg of vicilin. At the same concentration of pentanal, about 15 nmoles of pentanal were bound per mg of vicilin. Taking into account the total amounts of aldehyde and protein present, this amount of octanal bound to vicilin is about 96% of the total amount of octanal added, whereas for pentanal about 52% of the total amount of pentanal added was bound (**Table 1**). Pentanal thus has a lower affinity for vicilin than octanal.

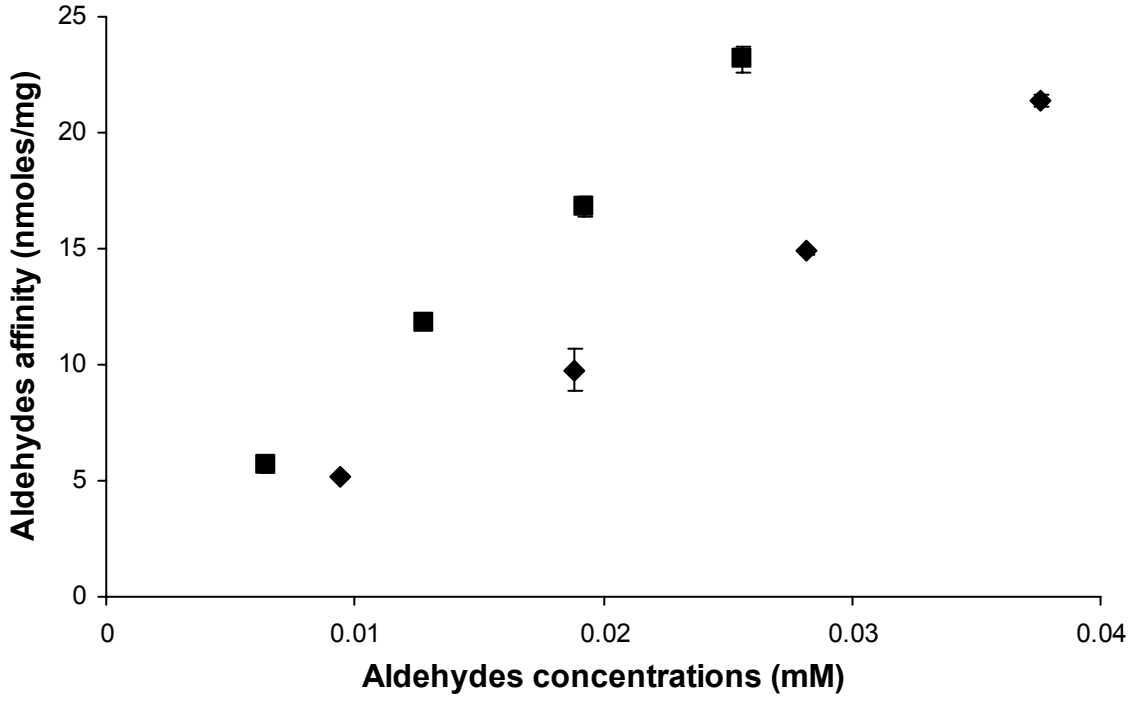


Figure 1: The interaction of 0.1% (w/v) vicilin with octanal (■) and pentanal (♦) at pH 7.6. Error bars indicate standard deviations (n=3).

Table 1: The amount of aldehydes and ketones bound to vicilin at a concentration of 0.025 mM, heated and non-heated fractions, at pH 7.6.

Volatile	Octanal affinity (nmoles/mg)	% bound[#]
Vicilin non-heated		
Octanal	~23	96
Pentanal	~15	52
2-Octanone	<1.5	33
2-Heptanone	<1	19
2-Hexanone	<1	13
2-Pentanone	<0.5	10
Vicilin heated		
Octanal	~9	32
2-Octanone	<0.5	16

[#]Calculated as a proportion of the total volatiles added to the system

The amounts of 2-pentanone, 2-hexanone, 2-heptanone and 2-octanone bound to vicilin at pH 7.6, at concentrations of 0.02-1.16 mM, are shown in **Figure 2**. The amount of binding for both aldehydes and ketones also increased with increasing VOCs concentrations. It can be seen that compared to the aldehydes, the ketones are bound with a much lower affinity. The amount of ketones bound to vicilin is on average 10-33% of the total amount added (**Table 1**). Similar to the aldehydes, the ketones with a longer chain-length have higher affinities to vicilin than those of lower chain-length. The results obtained are in agreement with other protein-VOC interaction studies, which also showed that binding increased with increasing VOCs concentrations (King & Solms, 1979; Landy *et al*., 1995; Hansen & Booker, 1996), and with increasing chain length (Damodaran & Kinsella, 1980; 1981a/b; 1983; O'Neill & Kinsella, 1987b; Andriot *et al*., 2000). It should be noticed that the concentrations of VOCs added to the protein in this study are relatively low (maximum 105 ppm), and most of the added VOCs was bound to the protein. As in actual food production, even lower concentrations in parts per million (ppm) to parts per trillion (ppt) of VOCs are added, it can be expected that in those products most of the added flavour is bound to other food ingredients. Most authors, however, have studied VOCs interactions at relatively high concentrations (500 to 10,000 ppm) (Landy *et al*., 1995; Druaux *et al*., 1995; Li *et al*., 2000), which are not realistic for use in food processing, and may thus lead to an overestimation of the amount of free VOCs. In addition, the solubility of these VOCs used in these studies may not be completely soluble at such high concentrations.

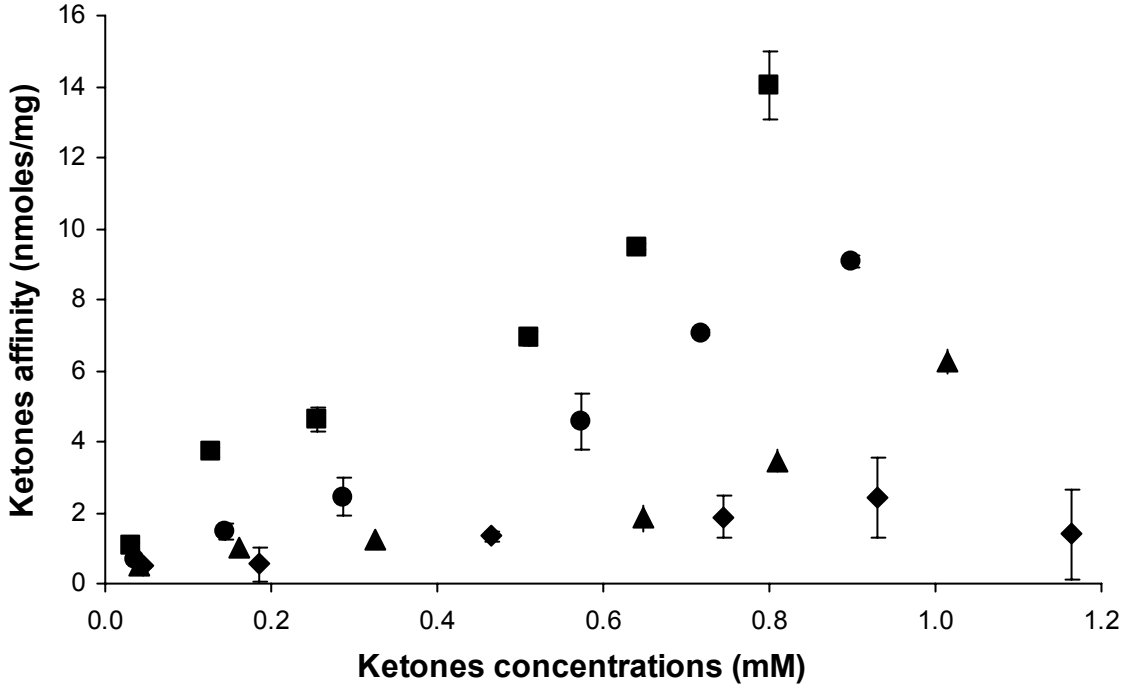


Figure 2: The interaction of 1% (w/v) vicilin with 2-octanone (■), 2-heptanone (●), 2-hexanone (▲) and 2-pentanone (♦) at pH 7.6. Error bars indicate standard deviations (n=3).

Effect of heat on protein-flavour interactions

The effect of heating on vicilin-VOC interactions was only studied with 2-octanone and octanal because the ketones and aldehydes of lower chain length showed no binding to vicilin after heating (results not shown). From **Figures 3 and 4**, it can be seen that the amounts of binding of both octanal and 2-octanone to vicilin decreased upon heat treatment. The amount of octanal bound to non-heated vicilin was about 96% of the total amount of octanal present, whereas the amount of octanal bound to heated vicilin was only 32% (**Table 1**). The amounts of 2-octanone bound to non-heated and heated vicilin were about 33% and 16% of the total amount of added 2-octanone, respectively.

Heating is known to cause protein denaturation, which is often followed by aggregation. This aggregation might lead to a reduction of total exposed surface area of the protein, and may hence reduce the observed VOCs binding. In order to give an explanation for the flavour binding properties of heated vicilin using this reduction in exposed surface area, the molecular size of vicilin

molecules before and after heating was estimated by using gel filtration chromatography. From the results obtained (results not shown), it was estimated that aggregates of at least 4200 kDa, *i.e*. 83 monomers of vicilin (50 kDa), were formed after heating vicilin at 90 ^{o}C for 30 min. The exposed surface area of both the non-heated vicilin and the aggregated protein after heating was estimated by assuming both particles to be spheres. The radius of a sphere-like protein can be estimated using the formula: $4/3\pi r^3 = 1.27$ * Molecular weight (in $Å^3$) (Creighton, 1993). Using this formula, the surface area of such a protein can be estimated. The surface area of an aggregate of 83 molecules of vicilin monomers (50 kDa) would then be approximately 77% smaller than that of 83 vicilin molecules that are not aggregated. From the results shown in **Figures 3 and 4**, it can be seen that the amount of binding of 2-octanone and octanal to vicilin after heating decreased by 17% and 64%, respectively. These decreases in amount of binding are less than the estimated 77% decrease in surface area. Heating may thus have led to a decrease in available binding area but it does not necessarily decrease the affinity of the protein. An increase in the binding affinity for hydrophobic VOCs is often observed upon heating (Arai *et al*., 1970; Damodaran & Kinsella, 1981a; O'Neill & Kinsella, 1988; Chung & Villota, 1989; Druaux *et al*., 1995; Hansen & Booker, 1996), but our results do not give specific indications for such behaviour.

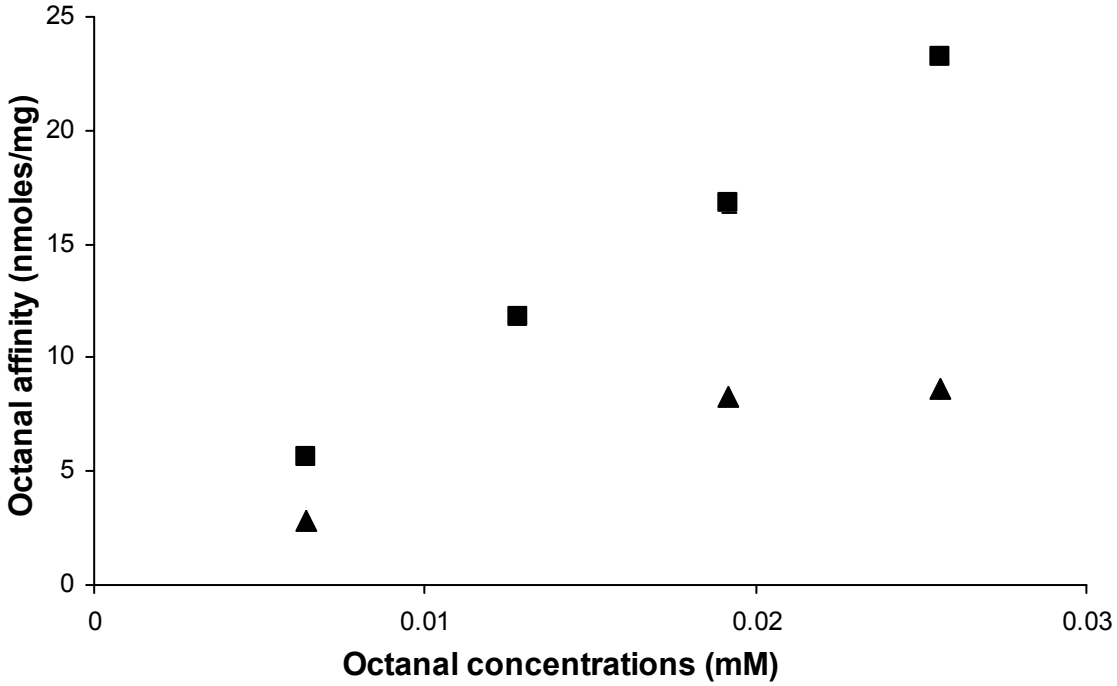


Figure 3: Effect of heat on the interaction of 0.1% (w/v) vicilin with octanal at pH 7.6. Non-heated vicilin (■) and heated (90 ^{o}C, 30 min) vicilin (▲) are presented.

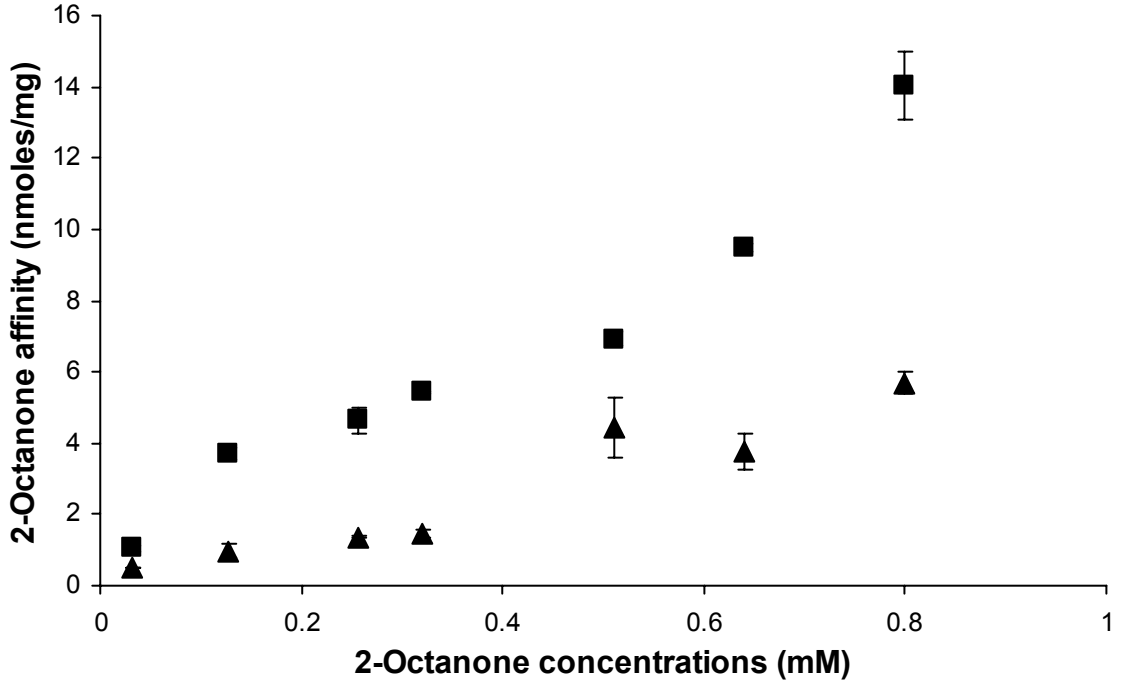


Figure 4: Effect of heat on the interaction of 1% (w/v) vicilin with 2-octanone at pH 7.6. Non-heated vicilin (■) and heated (90 ^{o}C, 30 min) vicilin (▲) are presented. Error bars indicate standard deviations (n=3).

Effect of pH on flavour binding: Difference between the soluble and the insoluble vicilin fractions

To investigate the effect of pH on binding of volatile flavour compounds, vicilin was subjected to pH values of 4.5, 5.5 and 6.5. At all these pHs, part of the vicilin became insoluble. The proportions of protein in the pellets and supernatants of a 0.2% (w/v) vicilin preparation, at various pHs are shown in **Figure 5a**. It can be seen that the pellets at pH 4.5 and especially at pH 6.5, contained relatively low amounts of protein (17% and 6%, respectively), whereas the pellet at pH 5.5 (the isoelectric pH of vicilin; Derbyshire *et al*., 1976) contained much more protein (61%). The soluble and insoluble vicilin fractions at each pH were used for interaction studies with octanal (25 μM). The binding affinities of the pellet and supernatant fractions at the various pHs are shown in **Table 2**. It can be seen that all pellets had higher binding affinities for octanal than the supernatants at the same pH. The pellet at pH 6.5 showed, for instance, a 10 times higher binding affinity than the supernatant at pH 6.5.

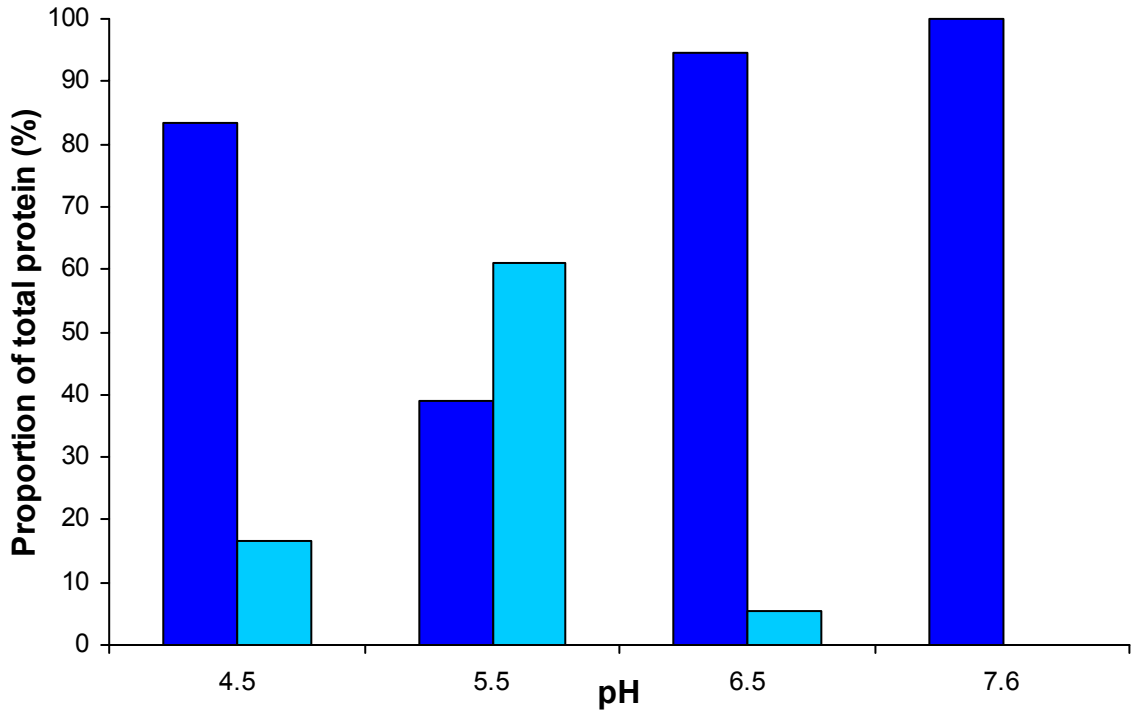
Table 2: Amount of octanal bound to vicilin pellets and supernatants at various pH.

	Octanal affinity (nmoles/mg of protein)					
	Non extracted		Hexane extracted		Ch:MeOH extracted	
pH	Supernatant (+/-SD)	Pellet (+/-SD)	Supernatant (+/-SD)	Pellet (+/-SD)	Supernatant (+/-SD)	Pellet (+/-SD)
4.5	5.8 (0.5)	23.5 (2.3)	2.3 (0.4)	27.4 (1.2)	1.4 (0.2)	18.6 (1.1)
5.5	4.8 (0.5)	6 (0.9)	6.5 (0.3)	8.3 (1.5)	5.8 (0.5)	8.7 (0.7)
6.5	4.4 (0.4)	41.4 (1.2)	5 (0.5)	40.1 (1.8)	4.9 (0.2)	19.3 (1)
7.6	22.1 (0.9)	-	19.7 (0.8)	-	9.3 (0.9)	-

Ch:MeOH stands for chloroform:methanol (1:1) extraction. SD is the standard deviation of triplicate samples

Both the proportions of total protein and the binding affinities of the supernatant and pellet fractions are shown in **Figure 5a/b**. It can be seen that the supernatant fractions, which contained a large proportion of the protein (**Figure 5a**), had a low binding affinity (**Figure 5b**). Interestingly, the pellet fractions contained only a low proportion of the protein present but possess a very high binding affinity. This high binding affinity was negatively correlated with the amount of protein in the pellet, which indicates that non-protein components presumably dominated octanal binding to the pellet material.

(a)



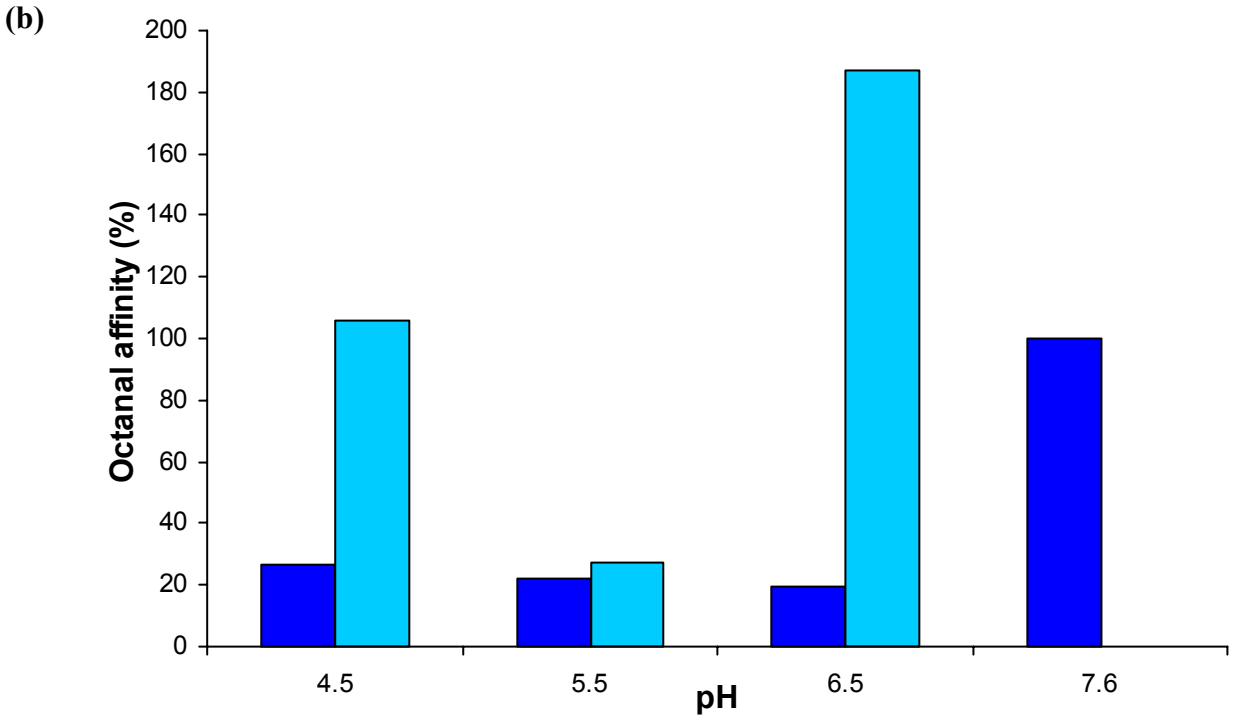


Figure 5: The proportion of protein **(a)** and octanal binding affinity **(b)** in supernatants ( ) and pellets ( ) of vicilin at various pH. The proportion of protein and octanal binding affinity are expressed as proportion of the total amount of protein and the binding affinity at pH 7.6, respectively.

Extraction of non-protein components

(i) Hexane extraction

In order to investigate the presence of binding of flavour compounds of non-protein components, the pellets were analysed for the presence of lipids and carbohydrates, which are known to bind flavour molecules (Godshall & Solms, 1992; Godshall, 1997; Plug & Haring, 1993; 1994; Hatchwell, 1994; de Roos, 1997; Rogacheva *et al*., 1999; Guichard, 2002). **Table 3** shows that both lipids and carbohydrates were found in the untreated vicilin pellets in amounts that could be responsible for the observed flavour binding.

Table 3: Amounts of lipid and carbohydrate of insoluble vicilin fractions at various pHs.

	Amount in pellet (μg)					
	Non extracted		Hexane extracted		Ch:MeOH extracted	
pH	Lipids (+/-SD)	Carbohydrates (+/-SD)	Lipids (+/-SD)	Carbohydrates (+/-SD)	Lipids (+/-SD)	Carbohydrates (+/-SD)
4.5	151 (10)	334 (15)	112 (5)	66 (2)	28 (7)	11 (4)
5.5	248 (31)	592 (15)	192 (14)	99 (4)	43 (4)	10 (3)
6.5	96 (6)	126 (12)	93 (13)	43 (4)	26 (4)	18 (6)

Ch:MeOH stands for chloroform:methanol (1:1) extractionSD is the standard deviation of triplicate samples

Next, the vicilin preparation was subjected to hexane extraction to remove the lipids. The amount of lipids in the pellets decreased by about 25% after hexane extraction, except in the pellet formed at pH 6.5 (**Table 3**). Similarly, the total amount of carbohydrates in the pellets also decreased substantially after hexane extraction (**Table 3**). The decrease in the amount of carbohydrates was larger than the decrease in the amount of lipids. The decrease in the amount of lipids and the efficient removal of carbohydrates upon hexane extraction, however, did not lead to a decrease in the binding affinity of the pellets (**Table 2**).

(ii) Chloroform/methanol extraction

As the hexane extracted vicilin pellets still contained a relatively high amount of lipids, and the amount of binding of volatile flavour compounds had not decreased upon extraction with hexane (**Table 2**), it was speculated that polar lipids, which are not extracted by hexane, might be present. Hence, the hexane extracted pellets were further extracted with chloroform/methanol (1:1). It can be seen in **Table 3** that the amount of lipids in the pellets decreased on average by about 75% upon chloroform/methanol (1:1) extraction. Chloroform/methanol (1:1) extraction also removed most of the carbohydrates that remained after hexane extraction. The removal of lipid components using the chloroform/methanol extraction led to a decrease in octanal binding, as observed for the pellets obtained at pH 4.5 and pH 6.5 (**Table 2**), suggesting that the presence of polar lipid components might be partly responsible for the high binding affinity for octanal. The decrease in binding affinity was more pronounced with the pellet obtained at pH 6.5 than with the pellet obtained at pH 4.5. There was no significant change in the binding affinity of the pellet obtained at pH 5.5, upon chloroform/methanol extraction, indicating that the pellet obtained at pH 5.5 had a composition different from those obtained at pH 4.5 and 6.5. Although chloroform/methanol extraction did reduce the flavour compounds binding affinity, an apparent binding affinity of as low as 1.4 moles/mg protein should be possible (**Table 2**).

Possible presence of polar lipids

The affinity for octanal seemed to be correlated to carbohydrate content as well as to the lipid content (**Table 2**). The lipids present consisted mainly of C18 fatty acid chains (results not shown), the content of which seemed to correlate with octanal binding. Possible compounds in peas that are known to be rich in C18 fatty acids are polar lipids such as galactolipids. There are 2 predominant classes of galactolipids in higher plants, the monogalactosyldiacylglycerols (MGDGs) and the digalactosyldiacylglycerols (DGDGs) (Dormann & Benning, 2002). MGDGs in general constitute up to 50% of polar lipids, whereas DGDGs constitute about 20% (Slabas, 1997). The most abundant class of fatty acids attached to the galactolipids is long chain polyunsaturated fatty acids *e.g.* α-linolenic acid (C18) (Dormann & Benning, 2002). It can be seen in **Table 3** that the weight ratio between the removed amounts of lipid (*e.g.* 149 μg) and the amount of carbohydrate (*e.g.* 89 μg), upon chloroform/methanol extraction of hexane treated material, is about 2, for all the pellets. The molar ratio between the non-polar moiety (glycerol and two C18 fatty acids; molecular weight = 620) and the 2 polar galactose units (Molecular weight = 324) in a DGDG is also approximately 2. These results may indicate that DGDG type galactolipids were initially present in the pellets and have been removed during chloroform/methanol extraction (**Table 3**), which has led to the observed decrease in the binding affinity of the pellets obtained at pH 4.5 and 6.5 (**Table 2**).

The vicilin preparation used had a higher binding affinity for aldehydes than for ketones. Although heat treatment of the protein resulted in an overall reduction in binding of flavour compounds, this could not be ascribed to a change in the affinity of vicilin for the VOCs. The insoluble vicilin fractions obtained at various pH contained non-protein components (lipids and carbohydrates), which had a higher binding affinity for octanal than the protein itself. This finding shows that the presence of impurities can have a significant influence on observed flavour binding. The presence of lipids and other non-protein components in protein preparations and their role in flavour binding should thus be investigated further.

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Chapter 7

General Discussion

The flavour of a food is an important aspect that contributes to a large extent to its acceptability and quality, which is also true for Novel Protein Foods (NPFs). The interdisciplinary program, PROFETAS, PROtein Foods Environment Technology And Society, was established with the aim of providing a toolbox, in which aspects of technology, environment and society are addressed. Flavour aspects were part of the technological aim to provide knowledge on the production of NPFs using green peas. Little was known about the endogenous flavour composition of peas and its protein preparations. In this thesis, both volatile and non-volatile pea flavour compounds were investigated. For volatiles, the presence of endogenous volatile organic compounds (VOCs) and the interaction of exogenous VOCs with pea proteins were explored. A number of exogenous VOCs that resembles those in peas were chosen for interaction studies so as to study retention of flavour compounds by pea proteins. The influence of heating and the presence of non-protein components, *e.g*. lipids, on these interactions were investigated. For non-volatiles, the presence of saponins in pea flour, and their perceived sensory characteristics, and stability, were studied.

(A) VOLATILES

Affinity of pea proteins for VOCs

Pea vicilin and legumin have shown to differ in their affinities for VOCs as suggested by their endogenous VOC concentrations (Chapter 5). The interaction of pea vicilin with exogenous VOCs was studied further in Chapter 6, as it showed greater VOC interaction capacity than pea legumin. Pea legumin (~60 kDa) was found to have affinity for aldehydes only (**Figure 1**). At 0.13 mM of octanal, about 70 nmoles of octanal were bound per mg of legumin and at 0.18 mM of pentanal, about 50 nmoles of pentanal were bound per mg of legumin, constituting ~51% and ~28% of the total amount of octanal and pentanal added to the system, respectively. Compared to vicilin, legumin retains much less aldehyde at the same aldehyde concentration (At 0.03 mM octanal, vicilin retains ~3500 nmoles/mg, whereas legumin retains ~16 nmoles/mg). From Chapter 5 (Table 2), it was observed that legumin contained substantially more VOCs (in arbitrary units; AU), noticeably aldehydes and alcohols, than vicilin. This may indicate that legumin has substantially higher affinity than vicilin and might have endogenous VOCs occupying its available interaction sites. As a result, a legumin preparation might have little or no retaining capacity for added aldehydes. In order to make a better comparison on the affinities for flavour retention of legumin and vicilin, both proteins should ideally be free of any VOCs before interaction studies.

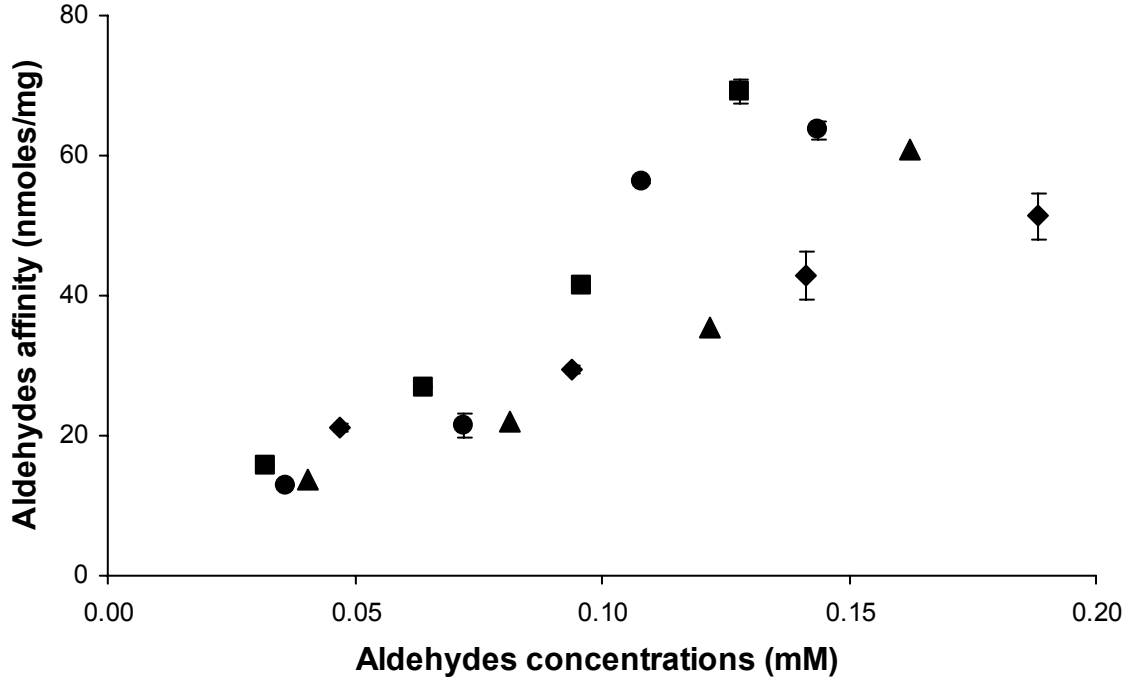

Figure 1: The interaction of 0.1% legumin with aldehydes, octanal (■), heptanal (●), hexanal (▲) and pentanal (♦) at pH 7.6. Error bars indicate standard deviations.

Factors influencing the retention of VOCs

In addition to proteins, other components in a food matrix such as fats and carbohydrates tend to adsorb flavour compounds (Plug & Haring, 1993; 1994; Fischer & Widder, 1997), resulting in their retention (Chapter 5). The retained compounds in peas are mostly odour active VOCs (*e.g.* aldehydes, ketones and alcohols), which are degradation products of fatty acids that lead to peas being perceived as having an 'off-flavour' (Murray *et al*., 1976). For pea proteins to be used as ingredients, a manufacturer would have to take into consideration the types and quantity of the VOCs that are present in the pea flour and its protein preparations (Chapter 5), and their odour activities. For example, three 3-alkyl-2-methoxypyrazines have been identified as natural green pea flavour by several authors (Murray *et al*., 1970, 1976; Murray and Whitfield, 1975; Jakobsen *et al*., 1998). Although these components were not identified in our studies, in which dried green peas were used, they should not be overlooked because they are odour active compounds at minute quantities due to their low threshold values. The detection of these compounds by others may be due to the use of different types of peas, such as raw green peas (Murray and Whitfield, 1975), unblanched or blanched frozen peas (Murray *et al*., 1976; Jakobsen *et al*., 1998). The absence of these compounds could also likely be due to the treatments involved in the production of pea

protein preparations. In this thesis, the factors contributing to flavour retention were identified and discussed further.

(i) Effect of purification

The extraction of pea flour and the purification of pea proteins led to the removal or reduction in concentrations of some VOCs (Chapter 5; Table 2), which is a positive effect for pea flour and pea protein preparations to be used as 'flavour free' starting materials that would allow wider use of these ingredients in food production. Depending on the pH, the retention of VOCs in pea flour varies (Chapter 5; Table 1) with the class and the hydrophobicity of VOC. A higher amount of VOCs was observed to be released at pH 8 than at pH 4 (Chapter 5; Table 1) and the protein preparations (protein isolate, legumin and vicilin) contain a lower amount of total volatiles than the pea flour at the same pH (Chapter 5; Table 2). With respect to NPFs production, one would like to use the protein preparations that have a low amount of endogenous VOCs, *i.e*. less probability for 'off-flavour'. Nevertheless, the success of NPFs should also consider texture, in addition to flavour. From the work of O'Kane (2004), it is known that pea vicilin has the ability to form transparent gels, whereas pea legumin and protein isolate formed opaque gels, at minimum gelling concentrations of 14% (w/v), 10% (w/v) and 16% (w/v), respectively. As the production of NPFs involves the addition of many other ingredients, gel transparency would be of minor importance compared to the minimum gelling concentration. Although legumin requires the least amount of material in order to form a gel, it has the largest amount of endogenous VOCs (Chapter 5; Table 2) compared to vicilin or protein isolate. Vicilin has lower minimum gelling concentration than protein isolate, but it would require additional cost for further purification. Depending on the type of NPFs to be produced, pea vicilin may be an option because it has the minimum amount of components leading to off-flavour compared to legumin and protein isolate (Chapter 5; Table 2), whereas protein isolate has the advantage of more cost-effective preparation compared to vicilin. From the results obtained in this thesis, protein extraction from peas could be done preferably at pH 8, at which the removal of endogenous VOCs is evidently a positive benefit, however, it may be more economical to mask off-flavour by *e.g*. addition of exogenous VOCs.

(ii) Effect of non-protein components on pea vicilin-VOC interactions

The presence of lipids and carbohydrates and their influence on VOC interactions proved to be of significant importance (Chapter 6). Our results showed that the supposedly 'pure protein preparations' of vicilin contained other components, such as lipids and carbohydrates, which have

higher affinity for VOCs than the proteins themselves. Lipids may have a more predominant influence on flavour retention than carbohydrates (Godshall, 1997). The addition of vegetable oil has been shown to decrease the odour thresholds of aldehydes significantly (Buttery *et al*., 1973), indicating the interaction of lipids and aldehydes. It can be expected that the presence of lipids may also influence the retention of VOCs by other pea proteins, such as legumin and convicilin. Lipids may possibly be the reason for the retention of endogenous VOCs (Chapter 5) in the protein preparations, which may lead to 'off-flavours', if used for NPF production. Lipids themselves may also be 'off-flavours'. To remove such 'off-flavours', one may need to apply an efficient lipid extraction step prior to protein extraction and purification. Hexane extraction may not sufficiently remove lipids compared to chloroform/methanol extraction, as can be seen from Chapter 6. As chloroform/methanol is not food grade, this solvent cannot be used to remove (polar) lipids (and together with this, off-flavours) from materials to be used as food ingredients. Aqueous ethanol (*e.g*. 70%) could be used as an alternative, but the efficiency with respect to lipids/off-flavours removal remains to be established. In addition, this treatment may lead to a change in protein conformation, which may result in a protein that is not functional for gelling, and this requires further investigation.

In conclusion, the presence of VOCs in pea flours and pea protein isolates would be of greater importance than their presence in the pure protein preparations, as most industrial products contain these two ingredients. In addition, heating was found to cause a significant reduction in the amount of exogenous VOCs retained by vicilin (Chapter 6), which is likely due to a change in the protein's structure (*e.g*. aggregation). On the other hand, heat can also cause the release of endogenous VOCs (Chapter 5), giving a 'flavour free' protein with more industrial flexibility.

(B) NON-VOLATILES

Characteristics of pea saponins

Most of the saponins isolated from plants are reported to be bitter in taste, such as from peas (Price & Fenwick, 1984; Price *et al*., 1985; Heng *et al*., 2005). Pea saponins, namely DDMP saponin and saponin B, were found to be present in pea flour of various pea varieties (Chapter 3) with DDMP saponin being the predominating saponin. These saponins were found to be bitter even at concentrations as low as 2 mg/L, with DDMP saponin being significantly more bitter than saponin B (Chapter 3). DDMP saponin has a similar threshold level to the common bitter reference

compound, quinine sulphate, both are perceived at concentration < 2 mg/L, whereas that of saponin B is ~ 8 mg/L (Chapter 3). Moreover, saponins were also reported to have affinity for proteins (Potter *et al*., 1993; Ikedo *et al*., 1996; Shimoyamada *et al*., 1998, 2000; Morton & Murray, 2001; Liu *et al*., 2003) and were found to be present in pea protein isolates (Heng *et al*., 2004). Therefore, pea flours and pea protein isolates, which are used industrially as ingredients for non-meat products, *e.g*. NPFs (Davies & Lightowler, 1998), may be bitter and bitterness should preferably be removed or masked. Assuming that there are 0.2% (w/w) saponins in pea flour (Heng *et al*., 2005), which has a protein content of ~25% (Casey, 2003), and that all saponins end up in the protein isolate fraction during protein extraction, it can be calculated that the protein isolate contains approximately 0.8% (w/w) saponins. The protein isolate is used to make NPF, which requires a high amount of protein, *e.g*. 55% protein is used to make Tivall vegetarian frankfurter. This will result in 4.4 g of saponin per kg of the NPF product. Assume that during processing, all DDMP saponins are converted to saponin B (Chapter 2). The resulting NPF would have a threshold value of 4400 mg/kg, which is at least 500 times more than the threshold value of saponin B (8 mg/L = 8 mg/kg), and therefore the taste of the product will be very bitter. One may consider using pea varieties that contain relatively low amount of saponins (Chapter 3). The variety with the lowest saponin content is 3 times lower than the variety with the highest saponin content. This is, however, insufficient to overcome the much higher threshold present in the NPF. Therefore, to produce an NPF that has no bitterness and has an acceptable texture, saponins would preferably be removed; by *e.g*. 70% ethanol extraction. Alternatively, exogenous VOCs would have to be added to mask bitterness. It is also important to note that the results obtained in our studies (Chapter 2 and 3) were achieved from tests using pure saponin preparations that contained only DDMP saponin and saponin B. It is possible that the behaviour of DDMP saponin, which was observed to be unstable in the pure systems, and its taste perception, may be different within a complex food matrix containing other components *e.g*. proteins, and this is thus worth investigating further.

Saponin analysis

The bitterness of peas is related to their saponin contents, which is in turn dependent on the pea variety (Chapter 3). Several other studies have shown that pea varieties differ in saponin content (Bishnoi & Khetarpaul, 1994; Daveby *et al*., 1997), but the saponin contents reported in these studies vary greatly and often depend on the methods used for extraction and quantitative analysis (**Table 1**).

Table 1: Overview of saponin composition and content reported in several studies on peas, in which different methodology was employed for extraction and analysis

Materials	Total Saponin content (g/kg dry weight)	Ratio of B/DDMP	Extraction conditions	Method of quantification	Reference
Dried peas *Pisum sativum* *Pisum elatius* *Pisum arvense*	0.7-1.9	**0.2-0.6**	Room temperature, 1h with 70% ethanol	RP-HPLC-ELSD	Heng *et al*. (2005)
Green peas (*Pisum sativum*)	11	Not known	Soxhlet extraction 24h with methanol	TLC	Fenwick & Oakenfull (1983)
Dried peas (*Pisum sativum*)	0.1	Only B	20^{o}C, 1h with water; Freeze dried sample extracted at 20^{o}C, 1h with methanol	TLC	Price & Fenwick (1984)
Pea flour (*Pisum sativum*)	2	Only B	<15^{o}C, 0.5h with water (3X), freeze dried	TLC; GC	Price *et al*. (1985)
Pea flour (*Pisum sativum*)	1.8 (TLC); 1.4 (GC)	Only B	Soxhlet extraction 30h with methanol, evaporation to dryness	TLC; GC	Curl *et al*. (1985)
Dried green peas (*Pisum sativum*); yellow split peas (*Pisum sativum*)	1.8 (green peas); 1.1 (yellow split peas)	Only B	Soxhlet extraction 30h with methanol, evaporation to dryness	TLC	Price *et al*. (1986)
Dried green peas (*Pisum sativum*); yellow split peas (*Pisum sativum*)	1.8 (green peas); 1.1 (yellow split peas)	Only B	Soxhlet extraction 30h with methanol, evaporation to dryness	RP-HPLC-UV	Price *et al*. (1988)
Field peas; vegetable peas (*Pisum sativum*)	1.1 (field peas); 2.5 (vegetable peas)	Not known	50-60^{o}C, 12h with 80% ethanol	Colouring reagent, Lieberman-Burchard	Bishnoi & Khetarpaul (1994)
40 Swedish peas (*Pisum sativum*)	0.8-2.5	Only B	Soxhlet extraction 2h with 80% ethanol	RP-HPLC-UV	Daveby *et al*. (1997)
Green peas (*Pisum sativum*)	35	Only B	Soxhlet extraction 2h with methanol	RP-HPLC-UV	Kinjo *et al*. (1998)
Swedish peas (*Pisum sativum*)	Not quantified	**0.24 (0.5h); 0.55 (24h)**	Room temperature, 0.5-24h with 80% ethanol	RP-HPLC-UV	Daveby *et al*. (1998)

It can be seen from **Table 1** that other authors, who used TLC or RP-HPLC methods, reported the presence of only saponin B, except for Daveby *et al*. (1998). Although these authors used mild extraction conditions, they may have overlooked a critical factor that is necessary to maintain DDMP saponin stability (Chapter 2), and hence only found saponin B. Although the extractions used by these authors were done using Soxhlet refluxing (boiling) in the presence of ethanol or methanol, *i.e*. at conditions at which DDMP saponin may be expected to be stable (Chapter 2), the prolonged evaporation step, which is necessary after extraction to remove the ethanol or methanol, may have resulted in the release of all DDMP groups present.

The DDMP group is attached to C19 of saponin B aglycone (Chapter 4; Table 2) by an ether linkage, which is sensitive under various conditions (Chapter 2) and easily removed from the aglycone. It is possible that other groups attached to C19 (Chapter 4; Table 2) by the same linkage might also be easily detached from their aglycones under similar conditions mentioned in Chapter 2. The angeloyl group attached by an ether linkage to C19 (Chapter 4; Figure 5) of the aglycones, protoaescigenin or barringtogenol (Chapter 4; Figure 3; Skeleton 1), may likely detached under the extraction conditions of 20% methanol and boiling for 3 h (Voutquenne *et al*., 2002) and boiling in 20% methanol (Massiot *et al*., 1992). Under such extraction conditions, labile saponins may be relatively unstable (Chapter 2). Therefore, it would be necessary to use mild extraction conditions (Chapter 2) when isolating these saponins, to avoid underestimating their quantities.

In conclusion, the work done on saponins has provided a deeper understanding of saponins stabilities through the process of optimising the method of extraction. With the various conditions for DDMP stability known, the controversial issue of native saponin in peas was resolved. A reproducible method that extracts native saponin from peas also allowed the determination of DDMP saponin bitterness in comparison to saponin B. The relationship between saponin contents and bitterness has given a reason for the use of pea varieties with low saponin contents for food application.

Future exploration

The results achieved in this research have contributed to a better understanding on the behaviour of flavour compounds in pea and its protein preparations, which is helpful in exploring further use of peas as food ingredients, with the ultimate aim to produce pea products with acceptable aroma and

taste. Although pea proteins play a role in flavour retention, results from this thesis show that non-protein components may be of major importance in flavour retention. Therefore, their flavour retention ability and/or their removal without interfering with the properties of proteins should be explored further. The interaction of flavour compounds with pea flour and pea protein isolates, the most likely pea ingredients in NPFs production, should be further investigated.

Although not investigated, the possibility of saponins interfering with protein-VOC interactions should not be overlooked. There are several reports that have shown saponins interacting hydrophobically with proteins, such as bovine serum albumin (Ikedo *et al*., 1996), soy proteins (Shimoyamada *et al*., 1998) and β-lactoglobulin (Shimoyamada *et al*., 2000). This means that saponins may occupy the hydrophobic pockets on the proteins and thus may compete with VOCs for the same interaction sites, or create more sites on the proteins. The retention of added VOCs could, hence, be reduced or be enhanced due to the presence of saponins, which would be an interesting area to explore.

It is known that there are some saponins that are sweet. Chapter 1 shows that saponins containing different skeletons can have the same sensory property, and that saponins of the same skeleton can have different taste. This indicates that sweetness or bitterness is not skeleton-dependent, but that taste perception resides in the type of substituents (both sugar residues and other functional groups) attached to the skeletons, as well as the stereochemistry of the attached substituents. Consider the structures of sweet liquorice saponins in comparison to that of bitter saponin B (Chapter 1; Figure 2). To become sweet, saponin B would require a number of modifications, such as the addition of a carbonyl at C11. Nevertheless, it would already be sufficient to obtain a saponin with a neutral taste, which probably requires less conversion steps. The conversion of bitterness to neutral taste, which can possibly be obtained by fermentation, is another interesting aspect for further research.

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SUMMARY

This thesis forms part of the multidisciplinary program, PROFETAS, PROtein Foods Environment Technology And Society, which was established with the aims to provide knowledge on technological, environmental and societal aspects for the development of novel protein foods (NPFs) based on pea proteins. The aim of this thesis is to investigate the flavour aspects of peas and its protein fractions, which are potential ingredients for use in NPFs. Both volatile and non-volatile pea flavours were explored. For volatiles, an inventory of endogenous volatile organic compounds (VOCs) from pea flour and pea protein preparations was made, and the interactions of exogenous VOCs with pea proteins were studied. The influence of heat and the presence of non-protein components on flavour retention were also investigated. For non-volatiles, saponins in pea flour were identified and quantified, and their sensory characteristics and stability were studied.

Chapter 1 introduces pea and its composition, which include pea proteins as well as endogenous flavour components. Flavour properties and perception in general as well as the types of flavour components present in peas, namely the volatile organic compounds and the non-volatile saponins are described. Interactions of proteins with volatile and non-volatile components are discussed.

Chapter 2 illustrates a method designed for isolating native saponins from peas, and how temperature, pH and ethanol concentration influence the stability of the saponins. DDMP saponin and saponin B were found to be present in peas. With careful optimisation of an extraction method, DDMP saponin was isolated as the predominant saponin and was used for stability studies. DDMP saponin in water was observed to be unstable at temperatures > 30 ^{o}C, and at acidic and alkaline pHs, with an optimal stability around pH 7. The loss of the DDMP group at 65 °C was prevented at > 30% (v/v) ethanol. The breakdown reaction of DDMP saponin (to give saponin B) has an activation energy of ~49 kJ/mol, indicating a chemical reaction with moderate temperature dependence. The mechanism of DDMP saponin decomposition is suggested to consist of a fast protonation or deprotonation, followed by a rate-determining step in which maltol is the leaving group.

Chapter 3 presents the types and amounts of saponin in 16 pea varieties with the optimised method of Chapter 2, and shows the difference in bitterness of saponin B and DDMP saponin. Using a trained panel, DDMP saponin was found to be significantly more bitter than saponin B and the

perceived bitterness was found to correlate to saponin concentration. DDMP saponin has similar threshold level to the common reference bitter compound, quinine sulphate; both are perceived at a concentration of < 2 mg/L, whereas that of saponin B is ~ 8 mg/L. In addition, the 16 pea varieties investigated differed significantly in saponin content (0.7-1.9 g/kg; dry matter). DDMP saponin was more abundant than saponin B in all varieties; in two varieties, DDMP saponin was the only saponin present.

Chapter 4 gives an overview of the structures of plant saponins that have been isolated and characterised till present, and relates the structures to their distribution in the plant kingdom. The different cyclization mechanisms of oxidosqualene formed the basis of skeletal classification. A total of 21 skeletons were classified into 5 main classes of 66666, 66665A, 66665B, 6665A and 6665B, and the oleanane skeleton of group 66666 was found to be the most common skeleton present in most orders of the plant kingdom. An overview of the type of substituents and sugar chains attached to oleanane skeleton showed that the type of substituents and their position of attachment do not seem to be plant order-specific, and that sugar chains of 1 to 8 sugar residues can be attached to mainly C2 and/or C18 of the skeleton.

Chapter 5 gives an overview of the type and amount of volatile organic compounds (VOCs) released from pea flour, pea protein isolate and legumin and vicilin preparations. The effect of pH on the release of the volatiles from the pea flour and the effect of protein purification steps were investigated. There were 3 main groups of volatile organic compounds released from pea flour and its protein preparations: the aldehydes, ketones and alcohols, which differed in concentration. Both pH and protein purification were found to have a large effect on the amount and type of VOCs released, which could be explained by their log P values: VOCs with low log P values were extracted with aqueous solvents, whereas those with high log P values were retained in the protein preparations.

Chapter 6 describes the interaction of model VOCs (aldehydes and ketones) with pea vicilin at various concentrations. Vicilin was found to interact with both aldehydes and ketones at pH 7.6, and heating at 90 ^{o}C resulted in a decrease of these interactions. The insoluble fractions of vicilin preparations at pH 4.5, 5.5 and 6.5, which showed remarkably high affinities for octanal, were found to contain relatively high amounts of lipids and carbohydrates. The insoluble fractions at pH 4.5 and 6.5 showed a greater decrease in lipid than in carbohydrate contents after extraction with

various organic solvents. This decrease also led to a significant reduction in the retention of VOCs, indicating that lipids and carbohydrates have higher affinity than vicilin for the tested VOCs.

Chapter 7 discusses the factors affecting volatile flavour retention and the characteristics of pea saponins, and relates the importance of volatile and non-volatile flavour compounds of pea ingredients for use in the development of novel protein foods. This thesis encompasses substantial knowledge on endogenous and exogenous flavour aspects of pea flour and ingredients, which are essential for successful development of NPFs that have good taste.

SAMENVATTING

Het werk beschreven in dit proefschrift is onderdeel van het multidisciplinaire programma PROFETAS, PROtein Foods Environment Technology And Society. Het doel van PROFETAS was het ontwikkelen van innovatieve, eiwitrijke levensmiddelen op basis van erwteneiwitten, met speciale aandacht voor technologische, maatschappelijke en milieu implicaties. Dit proefschrift beschrijft de relatie tussen eiwitfracties uit erwten welke als potentiële ingrediënten voor NPF's zijn geïdentificeerd en hun impact op aroma. Zowel vluchtige als niet-vluchtige erwtenaroma's zijn onderzocht. Voor vluchtige aroma's werd een inventarisatie gemaakt van vluchtige organische componenten (VOC's) uit erwtenbloem- en erwteneiwitpreparaten. Tevens werden interacties tussen toegevoegde niet-erwten VOC's en erwteneiwitten bestudeerd. Daarnaast is de invloed van verhitting en de aanwezigheid van niet-eiwitcomponenten op aromabehoud onderzocht. Saponinen werden als niet-vluchtige componenten in erwtenbloem geïdentificeerd. Naast een kwantificering van saponinen in erwtenbloem werden de sensorische eigenschappen en de stabiliteit van een specifiek saponine-type bestudeerd.

In Hoofdstuk 1 werden erwten en de samenstelling van erwten inclusief erwteneiwitten geïntroduceerd. De eigenschappen van endogene aromacomponenten, waaronder de VOC's en de niet-vluchtige saponinen, worden beschreven. De interacties van erwteneiwitten met vluchtige en niet-vluchtige componenten worden bediscussieerd.

In Hoofdstuk 2 wordt een methode voor isolatie van natieve saponinen uit erwten toegelicht. Ofschoon de klasse der saponinen vele verschillende verbindingen omvat, bleek erwt slechts twee typen te bevatten, DDMP-saponine en saponine B. Het was bekend dat DDMP-saponine labiel is, en gemakkelijk omgezet kan worden tot saponine B door afsplitsing van de DDMP-groep. Met behulp van een zorgvuldig geoptimaliseerde extractiemethode kon DDMP-saponine worden geïdentificeerd als het meest voorkomende saponine in erwt.. De stabiliteit van de verkregen saponinen wordt onderzocht in relatie tot de temperatuur, pH en ethanolconcentratie. DDMP-saponine bleek instabiel te zijn in water bij temperaturen boven de 30°C en bij zure en basische pH-waarden, en had een maximale stabiliteit rond pH 7. Het verlies van de DDMP-groep bij 65°C trad niet op wanneer de ethanolconcentratie van het oplosmiddel groter was dan 30% (v/v). De activeringsenergie voor het uiteenvallen van DDMP-saponine is ongeveer 49 kJ/mol, hetgeen een

chemische reactie met een matige temperatuursafhankelijkheid suggereert. Het mechanisme van uiteenvallen van DDMP-saponine bestaat waarschijnlijk uit een snelle protonerings- of deprotoneringsreactie, gevolgd door een snelheidsbepalende stap met maltol als de vertrekkende groep.

In Hoofdstuk 3 wordt de geoptimaliseerde extractiemethode, beschreven in hoofdstuk 2, toegepast om saponinegehalte en –compositie in 16 erwtenrassen te bepalen. De 16 erwtenrassen waren significant verschillend in saponinegehalte (0.7-1.9 g/kg drooggewicht). In alle rassen was de concentratie DDMP-saponine groter dan die van saponine B. In twee varianten was alleen DDMP-saponine aanwezig. Tevens wordt het verschil in bitterheid tussen saponine B en DDMP-saponine beschreven. Met behulp van een getraind panel werd DDMP-saponine significant bitterder bevonden dan saponine B, en de waargenomen bitterheid correleerde aan de saponineconcentratie. DDMP-saponine heeft een vergelijkbare waarnemingsdrempel als de bittere referentiecomponent quininesulfaat. DDMP-saponine wordt reeds waargenomen bij een concentratie van 2 mg/mL, terwijl saponine B wordt waargenomen bij 8 mg/mL.

In Hoofdstuk 4 wordt een overzicht gegeven van de structuren van alle saponinen in planten die tot nu toe zijn geïsoleerd en gekarakteriseerd; de distributie van de verschillende structuren over het plantenrijk wordt beschreven. Saponinen worden in de plant gesynthetiseerd uit oxidosqualeen, een molecuul met 30 koolstofatomen. Dit nagenoeg lineaire molecuul kan op verschillende manieren gecycliseerd worden, onder invloed van zogenaamde cyclasen. De verschillende cyclisatiemechanismen van oxidosqualeen tot een koolstofskelet bestaande uit verschillende ringen vormde de basis voor onze classificatie van saponinen. In totaal werden 21 skeletten geclassificeerd binnen de volgende vijf hoofdgroepen; 66666, 66665A, 66665B, 6665A en 6665B. Het zogenaamde oleanaanskelet van groep 66666 bleek in vrijwel het gehele plantenrijk voor te komen. Een overzicht van de typen substituenten en de suikerketens verbonden aan het oleanaanskelet toonden aan dat het type substitutie en hun positie aan het skelet niet plantorde-specifiek waren. De suikerketens bestaande uit 1 tot 8 suikerresiduen waren voornamelijk gebonden aan C2 en/of C18 van het skelet.

In Hoofdstuk 5 wordt een overzicht gegeven van de typen en de concentratie vluchtige organische componenten (VOC's) vrijgemaakt uit erwtenbloem, erwteneiwitisolaat en legumine- en vicilinepreparaten. Legumine en viciline zijn de twee belangrijkste klassen van opslageiwitten in de

erwt. Drie hoofdgroepen VOC's werden geïdentificeerd. Dit zijn aldehyden, ketonen en alcoholen en ze verschillen in concentratie. De pH en de wijze van eiwitzuivering bleken beide een groot effect te hebben op de concentratie en het type van de vrijgekomen VOC's. De waarnemingen waren in overeenstemming met de log P-waarden (een maat voor polariteit) van de VOC's. VOC's met lage log P-waarden werden geëxtraheerd met waterige oplosmiddelen, terwijl VOC's met een hoge log P-waarden in de eiwitpreparaten werden behouden.

In Hoofdstuk 6 wordt de binding van model-VOC's (aldehydes en ketonen) aan erwtenviciline in diverse concentraties beschreven. Viciline liet een interactie zien met zowel aldehydes als ketonen bij pH 7.6. Verhitten bij 90°C vertoonde een afname van binding van VOCs aan viciline. Viciline bleek niet volledig oplosbaar te zijn bij verschillende pHs. Het onoplosbare deel bij pH 4.5, 5.5 en 6.5 had een opvallend hoge affiniteit voor octanal. Waarschijnlijk is dit te wijten aan een relatief hoge concentratie aan lipiden en koolhydraten. Na extractie met diverse organische oplosmiddelen vertoonden de onoplosbare fracties bij pH 4.5 en 6.5 een relatief grote afname in, met name, lipiden. De organisch oplosmiddel geëxtraheerde fracties lieten een significante reductie in de retentie van VOC's zien, hetgeen suggereert dat lipiden en koolhydraten een hogere affiniteit voor de geteste VOC's hebben dan het eiwit viciline.

Tenslotte worden in Hoofdstuk 7 de factoren beschreven die invloed hebben op de retentie van vluchtige aroma's en de eigenschappen van erwtensaponines. De toepassing van vluchtige en niet-vluchtige aromacomponenten uit erwteningrediënten worden bediscussieerd besproken in het kader van innovatieve eiwitlevensmiddelen. Dit proefschrift bevat substantiële kennis over endogene en exogene aroma-aspecten van erwtenbloem en -ingrediënten welke essentieel zijn voor een succesvolle ontwikkeling van sensorisch acceptabele NPF's.

概要

本课题是**PROFETAS(**蛋白质食品环境技术学会的缩写**)**的一个多学科项目，其立题目的是为了提供有关技术、环境、社会方面的知识，用来开发以豌豆蛋白为原料的新型蛋白质食品**(NPFs)**。本论文的目的是为了研究豌豆的风味特性及其蛋白质的成分。豌豆蛋白质是新型蛋白质食品的重要原料，我们研究了挥发性和非挥发性的风味成分。对于挥发性的物质来说，发现了豌豆粉和豌豆蛋白制备物内在的挥发性有机化合物，以及研究了豌豆蛋白的外在的挥发性有机化合物的相互作用，同时研究了加热和存在的非蛋白成分对风味保留的影响。对于非挥发性的物质来说，鉴定和测定了豌豆粉中的皂角甙含量，并对他们的感官特性和稳定性进行了研究。

第一章介绍了豌豆及其成分，包括豌豆蛋白及其内在的风味组成，存在于豌豆中的风味特征以及风味组成的类型，也称之为挥发性有机化合物和非挥发性的皂角甙，讨论了挥发性和非挥发性成分的相互作用。

第二章说明了一种方法，用来从豌豆中分离天然的皂角甙，研究了温度，**pH**值和乙醇浓度对皂角甙稳定性的影响。研究发现豌豆中存在着**DDMP**皂角甙和皂角甙**B**，选择适当的提取方法，分离得到的主要的皂角甙是**DDMP**皂角甙，并被用于稳定性研究。研究发现**DDMP**皂角甙在温度大于**30°C**的水中、在酸性和碱性条件下是不稳定的。在**pH 7**左右具有相当的稳定性，在**65°C**时，**30%**的乙醇溶液能防止**DDMP**皂角甙的损失。**DDMP**皂角甙的分解反应需要约**49KJ/mol**的活化能，表明该化学反应需要依赖于适当的温度。**DDMP**皂角甙分解的机制，可能包括快速的加氢或脱氢反应，以及影响其反应速率的其他步骤。

第三章，通过第二章的优化方法分离了**16**种豌豆中的皂角甙的类型及其含量，结果表明皂角甙**B**和**DDMP**皂角甙的苦味有一定的差异，通过感官小组评定**DDMP**皂角甙比皂角甙**B**更苦，并且其苦味的程度与皂角甙的浓度成正比。**DDMP**皂角甙和参照苦味化合物硫酸奎宁具有相似的阈值范围，二者浓度约小于**2mg/L**，而皂角甙**B**的阈值约为**8mg/L**。另外在**16**种豌豆中其皂角甙含量具有显着的差异性，其范围在**0.7**至**1.9g/kg**干基。在所有品种中**DDMP**皂角甙比皂角甙**B**含量丰富，其中有两个品种仅含有**DDMP**皂角甙。

第四章综述了现今已经分离和研究过的植物中皂角甙的结构特点，以及其结构与在植物中分布的相关性。不同的氧化循环机理形成了其主要分类的基础。共有21个碳原子组成的碳链骨架可以划分成5个主要的类别，分别为66666，66665A，66665B，6665A，6665B，其中66666骨架次序是植物王国中存在的最常见的结构。有关取代物的类型以及连接在碳碳骨架的糖基团的综述，表明取代物的类型以及连接在碳碳骨架的位置似乎不会影响植物特定的结构次序，并且1到8个糖残基形成的糖基团主要会连接在C2或C8的碳碳骨架上。

第五章概述了从豌豆粉、豌豆分离蛋白、豆类、球蛋白制备物等释放的VOCs(挥发性有机化合物)的类型和含量。研究了pH以及蛋白质纯化步骤对豌豆粉中挥发性物质释放的作用，从豌豆粉及其蛋白制备物中共检测到3组主要的VOCs，包括醛、酮和醇，他们的浓度是不同的。研究发现pH和蛋白质纯化对释放的VOCs的类型和含量影响较大，其可以通过LogP值解释，对于低的LogP值，VOCs可以用水溶剂萃取，而对于高的LogP值，VOCs则保留在蛋白制备物中。

第六章分析了典型VOCs(醛和酮)与不同浓度的豌豆球蛋白的相互作用。发现球蛋白在pH7.6可以与醛和酮发生相互作用，并且当加热到90°C会导致这种相互作用下降，在pH4.5、5.5和6.5时，在球蛋白制备中的不溶性组分具有对辛烷显着的亲和力，发现其中脂肪和碳水化合物含量较高。这些不溶性组分用不同的有机溶剂萃取后，在pH4.5和6.5时，在脂肪中的含量比在碳水化合物中的含量有显着的下降，这种下降还导致对VOCs保持力的极大减少，表明脂肪和碳水化合物比球蛋白对检测的VOCs具有较高的亲和力。

第七章讨论了延长豌豆皂角甙挥发性风味和特性的影响因素，并分析了用于开发新型蛋白质食品的豌豆配料中挥发性和非挥发性风味化合物的重要性。本课题需要解决有关豌豆粉及其配料内在的和外在的风味方面的实质性的问题，这对于我们成功开发具有良好滋味的新型蛋白质食品是必要的。

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I am sure you remember our saponin soap story
The brilliant idea that you came up so suddenly!
Cutting and pasting structures for several weeks daily
From a few hundred articles I got them ready

I had more than 80 pages documented
Showing it to you, you almost fainted!
Must shrink it down to 1 single page, you commanded
It has to be published and to be cited, you ordered!

Painstakingly, I began the process of shrinking
From 40 to 18 to 12 to 6...seems never ending...
Correction and discussion till 9pm in the evening
You were very helpful yet demanding...

At last! I got it down to a single page!
19 structures 'squeezed' almost to the edge
So much time and effort that we had engaged
I thought over the months a lot I had aged!

Thereafter we still had much discussion and revision
From 19 structures, one by one we had few more addition
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CURRICULUM VITAE

Lynn Heng was born on 6 March 1974 in Singapore. She did her studies in Singapore Polytechnic, first obtaining her Diploma in Chemical Process Technology, majoring in Food Technology in 1994, and went on to pursue an Advance Diploma in Food Technology, graduating in 1997. While doing her advance diploma studies, she was working as a Quality Control Analyst with a Flavour & Fragrance company, Takasago International (S). In addition, she was also a private tutor teaching Maths and Science. In 1997, she went to the University of Leeds, Procter Department of Food Science, and did a Master degree course in Food Science, graduating with distinction. Thereafter, she came to Wageningen (The Netherlands) and started her PhD at the Laboratory of Food Chemistry, working on a project of PROFETAS (PROtein Food Environment Technology And Society), and completed the research presented in this thesis.

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6. **Heng, L**. Book of PROFETAS. Chapter 3: Technological feasibility: Protein-flavour interactions. To be published.

Cover picture and layout by Alessandro and my sister, Bishan

Overview of completed training activities

Discipline specific activities

Courses

IOP Summer School of Industrial Proteins (Utrecht, June 2000)

Scientific Paper Writing (Amsterdam, May 2001)

VLAG course: *Advances Food Analysis* (Wageningen, March 2002)

Socrates Intensive Program: *Source of Raw Material for Industry* (Vienna, February 2003)

Applied Statistics by Dr. W. Hammers (Wageningen, 2002-2003)

Meetings

25th LOF Symposium (Brussels, February 2000)

3rd International Symposium on Industrial Proteins (The Hague, March 2001)

10th Weurman Flavour Research Symposium (Dijon, June 2002)

6th Lustrum Conference: *Food Sustainability* (Amsterdam, February 2003)

New Functional Ingredients and Foods: *Safety, Health and Convenience* (Copenhagen, April 2003)

4th International Symposium on Industrial Proteins: *Industrial Proteins in Perspective* (Ede, May 2003)

General courses

VLAG PhD week (Nijmegen, October 2000)

Food Chemistry PhD Study Trip (United States, October 2002)

Career Orientation Course (Wageningen, March 2003)

Optional

Preparation PhD research proposal

PROFETAS scientific meetings (2000-2004)

Centre for Protein Technology discussion group (2000-2004)

Printing: Ponsen & Looijen B. V., Wageningen